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<p><b>(54) Title:</b> MURINE INTRACISTERNAL A PARTICLE CONSTITUTIVE TRANSPORT ELEMENTS AND USES THEREOF</p> <p><b>(57) Abstract</b></p> <p>The invention provides for a novel post-transcriptional RNA nucleocytoplasmic transport element (NCTE), designated "CTE<sub>IAP</sub>," derived from a novel intracisternal A particle (IAP). The novel IAP was found inserted in a murine osteocalcinrelated gene (ORG) between its promoter and ORG's coding region, and is thus termed "IAP<sub>ORG</sub>". The invention also provides for an attenuated HIV-1 hybrid virus for use as a vaccine and a kit incorporating the hybrid virus. The kit also includes instructional material teaching the use of the vaccine, where the instructional material indicates that the vaccine is used for the prophylaxis or amelioration of HIV-1 infection in a mammal; that the vaccine is to be administered to a mammal in a therapeutically effective amount sufficient to express a viral protein; where the vaccine will not cause clinically significant CD4<sup>+</sup> cell depletion; and, the expression of the viral protein elicits an immune response to the attenuated HIV-1 virus. The invention further provides for a method for screening for a post-transcriptional RNA nucleo-cytoplasmic transport element (NCTE) binding protein.</p>		

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## MURINE INTRACISTERNAL A PARTICLE CONSTITUTIVE TRANSPORT ELEMENTS AND USES THEREOF

### FIELD OF THE INVENTION

This invention pertains to the field of virology and vaccine development. In particular, this invention pertains to the discovery that a novel post-transcriptional RNA nucleo-cytoplasmic transport element (NCTE) from a novel murine intracisternal-A particle (IAP) can be used to construct an attenuated retrovirus.

### BACKGROUND OF THE INVENTION

Intracisternal A-type particles (IAPs) are defective endogenous retroviruses found in a variety of mammals. These "particles" were first discovered because some murine IAPs with reverse transcriptase activity and polyadenylated RNA accumulate in the endoplasmic reticulum. Wilson (1974) *Biochemistry* 13:1087-1094; Leuders (1977) *Cell* 12:963-972. IAP retroviral genomes share extensive sequence homologies with D-type retroviruses. IAP genes in variable copy number, up to 2000 in a genome, exist in all rodent species studied and are dispersed throughout the genome. Because of this high copy number, IAP elements have been used as genetic markers. Kaushik (1994) *Mamm. Genome* 5:688-695.

Murine IAPs are constitutively expressed in high levels in many mouse tumors and at basal levels in most normal adult mouse tissues. Kuff (1990) *Cancer Cells* 2:398-400. While they are not infectious retroviruses, IAPs can be transcriptionally active. Expression of some murine IAPs results in translation products corresponding to retroviral gag and Pol polyproteins. Fehrmann (1997) *Virology* 235:352-359. Under certain circumstances, IAPs can transpose to different positions in the genome, in some cases acting as insertional mutagens. When these transpositions affect the expression of a gene that can either positively or negatively regulate cell growth, a malignant or transformed cell or tumor may arise. IAP transpositions have been frequently observed in hematopoietic

cells, often affecting cytokine gene expression. Wang (1997) *Cytokines Cell. Mol. Ther.* 3:3-19.

Genetic elements that possess IAP transposable characteristics are also contained in the human genome. Human IAP-like retroviral particles have been detected in a lymphoblastoid cell line exposed to mononuclear cells from a patient with severe CD4<sup>+</sup> T-cell deficiency who was negative for known human immunodeficiency virus (HIV) and human T-lymphotropic virus (HTLV) infection. Ultrastructurally, the human IAP particle was distinct from HIV and HTLV. Supernatants of activated mononuclear cells showed significant reverse transcriptase activity. While the patient's serum was negative for antibodies to known HIVs and HTLVs, his serum was positive for antibodies against the human IAP. Similar IAP particles were detected in a lymphoblastoid cell line exposed to mononuclear cells from the patient's daughter, who also showed CD4<sup>+</sup> T-cell dysfunction. These results suggest that human IAP is associated with CD4<sup>+</sup> T-cell immunodeficiency and dysfunction (Gupta (1992) *Proc. Natl. Acad. Sci. USA* 89:7831-7835).

Some retroviruses, including HIV type 1 (HIV-1), simian retrovirus type 1 (SRV-1), SRV-2, and Mason-Pfizer monkey virus (MPMV) (the later three are type D simian retroviruses), regulate their expression by controlling the nuclear transport of unspliced mRNA encoding structural proteins. They do this using *cis*-acting post-transcriptional RNA nucleo-cytoplasmic transport elements ("NCTEs," designated "CTEs" for some type D retroviruses). NCTEs fold into highly structured secondary structures, typically stem-loop structures. *Trans*-acting proteins bind to the NCTE loop structures to regulate RNA expression at several levels. Interaction of the NCTE with a *trans*-acting RNA binding protein stabilizes certain unspliced viral transcripts. This interaction is necessary for the message's transport from the nucleus to the cytoplasm and is necessary for expression of the transcript. Some retroviruses, such as Simian type D retroviruses, including SRV-1, do not encode their own *trans*-acting, NCTE-binding proteins and instead utilize cellular NCTE binding proteins. Other retroviruses, such as HIV-1, utilize a retrovirally-encoded NCTE RNA binding protein, called "Rev." HIV-1 regulates the expression of its structural proteins encoded by the *gag/pol*- and *env*-encoding transcript using this NCTE system. HIV-1's NCTE binding protein "Rev" interacts with a specific

NCTE sequence, designated the "Rev-responsive element," or "RRE," contained in its *gag/pol* and *env* encoding transcript. HIV-1's RRE does not bind cellular NCTE-binding proteins. Rev interacts directly with RRE as part of the RNA export machinery which transports RRE-containing transcripts to the cytoplasm from the nucleus. As a result, Rev and RRE are needed to produce infectious virus.

Simian retroviruses contain an NCTE, or "CTE," element located between their *env* gene and 3' long terminal repeat (LTR). HIV-1 lacking a functional Rev/RRE control system is uninfec̄tious, but can be reconstituted with simian retroviral CTE (*e.g.*, CTE from SRV-1, SRV-2, MPMV) to produce transcripts and infectious virions (Bray (1994) *Proc. Natl. Acad. Sci. USA* 91:1256-1260; Tabernero (1996) *J. Virol.* 70:5998-6011; Zolotukhin (1994) *J. Virol.* 68:7944-7952). However, reconstitution of RRE/Rev with CTE produces hybrids with lower levels of transcript and productive virion than seen with wild-type HIV-1.

It has been found that some individuals are infected with the HIV-1 virus but do not go on to manifest the symptoms of acquired immune deficiency syndrome (AIDS). Rev gene mutations that caused viral growth attenuation were found in some of these asymptomatic HIV-1 infected individuals. Thus, it was hypothesized that the resultant production of relatively low levels of virus contributed to their lack of disease progression, an asymptomatic state, and stable CD4<sup>+</sup> levels (Iversen (1995) *J. of Virology* 69:5743-5753; Hua (1996) *Virology* 222:423-429).

Thus, disruption of either the *cis*- (*e.g.*, CTE, RRE) or *trans*-acting (*e.g.*, Rev protein) elements of the RNA post-transcriptional nucleo-cytoplasmic transport regulatory system in retroviruses produces an attenuated virus, decreasing their replicative ability and pathogenicity *in vivo*. Such attenuated infection, results in an asymptomatic state in the infected individual. Thus, there exists a need for *cis*- and *trans*-acting post-transcriptional RNA regulatory elements capable of attenuating a retrovirus, such as HIV-1. The present invention fulfills this and other needs.

## SUMMARY OF THE INVENTION

The invention provides for an isolated nucleic acid consisting of a Constitutive Transport Element derived from an IAP nucleotide sequence, called CTE<sub>IAP</sub>.

The isolated CTE<sub>IAP</sub> nucleic acid is defined as having the following properties: (i) the CTE<sub>IAP</sub>, when inserted in a recombinant, hybrid HIV-1, is capable of functioning as a NCTE in place of wild-type NCTE in the hybrid HIV-1, and when the CTE<sub>IAP</sub>-containing hybrid HIV-1 virus infects activated human peripheral blood mononuclear cells

(huPBMCs), the level of expression of HIV-1 p24<sup>gag</sup> is between about 50 fold and about 200 fold less than levels of p24<sup>gag</sup> expression when HIV-1 wild type virus, utilizing wild-type NCTE, infects activated huPBMCs; and, (ii) the secondary structure of the CTE<sub>IAP</sub> comprises at least a two loop A domain and a loop B domain, wherein the nucleic acid primary sequence of the loops has at least 90% nucleic acid sequence identity to a loop A and a loop B domain of a nucleic acid comprising the loop structure as set forth in Figure 1 and the sequence as set forth in SEQ ID NO:1; and, the distance between the loop A domain and the loop B domain is between about 11 to about 36 base pairs in length.

In further embodiments, the invention provides for an isolated nucleic acid CTE<sub>IAP</sub> comprising a ribonucleic acid comprising a motif of contiguous base pairs consisting of X<sup>A</sup><sub>10-500</sub> - GUC AAUGAC GGGU AAGA - X<sup>B</sup><sub>16-24</sub> - ACCU AAGACA GG - X<sup>C</sup><sub>6-100</sub> - CAA U GUU - X<sup>D</sup><sub>6-100</sub> - CC GAGGAC AGGU - X<sup>E</sup><sub>16-24</sub> - CA ACCU AAGACA GGCA - X<sup>F</sup><sub>10-500</sub>, wherein X is any nucleotide base; and, an isolated nucleic acid CTE<sub>IAP</sub> comprising a deoxyribonucleic acid comprising a motif of contiguous base pairs consisting of X<sup>A</sup><sub>10-500</sub> - GTC AATGAC GGGT AAGA - X<sup>B</sup><sub>16-24</sub> - ACCT AAGACA GG - X<sup>C</sup><sub>6-100</sub> - CAA T GTT0 - X<sup>D</sup><sub>6-100</sub> - CC GAGGAC AGGT - X<sup>E</sup><sub>16-24</sub> - CA ACCT AAGACA GGCA - X<sup>F</sup><sub>10-500</sub>, wherein X is any nucleotide base. In other embodiments, the isolated nucleic acid CTE<sub>IAP</sub> comprises the sequence as set forth in SEQ ID NO:1; and, in the isolated nucleic acid CTE<sub>IAP</sub> the distance between the loop A domain and the loop B domain is between about 21 to about 26 base pairs in length. The invention also provides for an isolated transcription product of a CTE<sub>IAP</sub> nucleic acid, the CTE<sub>IAP</sub> nucleic acid as defined above.

The invention also provides for an expression vector comprising a nucleic acid encoding a CTE<sub>IAP</sub> and a non-naturally occurring nucleic acid sequence, the CTE<sub>IAP</sub> nucleic acid as defined above, and, a transfected cell comprising a polynucleotide encoding a CTE<sub>IAP</sub> and a non-naturally occurring nucleic acid sequence, the CTE<sub>IAP</sub> nucleic acid as defined above.

In another embodiment, the invention provides for a recombinant retrovirus either lacking or having a non-functional endogenous NCTE and further comprising a CTE<sub>IAP</sub> operatively inserted into the retrovirus and capable of acting as a functional exogenous NCTE to reconstitute the lacking or non-functional endogenous NCTE and to reconstitute the infectivity of the retrovirus in a mammalian cell, the CTE<sub>IAP</sub> as defined above. In alternative embodiments, the recombinant virus can be HIV-1, the NCTE can be RRE, and the HIV-1 can further lack a functional Nef.

The invention further provides for a vaccine for the prophylaxis or amelioration of a viral infection in a mammal comprising an attenuated retrovirus, where the retrovirus, when administered as a vaccine in sufficient amounts, is capable of eliciting an immune response to the retrovirus in a mammal with a functional immune system; and the attenuated retrovirus lacks an endogenous functional NCTE and/or the ability to express an endogenous functional NCTE binding protein, and the attenuated retrovirus further comprises a CTE<sub>IAP</sub> nucleic acid as defined above. In alternative embodiments, the attenuated retrovirus can HIV-1, the attenuated HIV-1 can also lack a functional Nef, the NCTE can be RRE, and the NCTE binding protein can be Rev.

Another embodiment provides for a kit for the prophylaxis or amelioration of a virus infection in a mammal, the kit comprising the above described vaccine and a pharmacologically acceptable carrier. The kit can further comprise an instructional material teaching the use of the vaccine, where the instructional material indicates that the vaccine is used for the prophylaxis or amelioration of HIV-1 infection in a mammal; that the vaccine is to be administered to a mammal in a therapeutically effective amount sufficient to express a viral protein; wherein the vaccine will not cause clinically significant CD4<sup>+</sup> cell depletion; and, the expression of the viral protein elicits an immune response to the attenuated HIV-1 virus.

The invention also provides for use of a CTE<sub>IAP</sub> in the manufacture of a medicament for the prophylaxis or amelioration of a viral infection, such as an HIV-1 infection, where the CTE<sub>IAP</sub> is as described above.

In one embodiment, the invention provides for a method for eliciting an immune response to a virus in a mammal, comprising administering to a mammal a

therapeutically effective amount of an attenuated recombinant virus, wherein the virus comprises the CTE<sub>IAP</sub> as defined above.

In another embodiment, the invention provides for a method for screening for a NCTE binding protein comprising the following steps: a) providing a composition comprising a loop A and/or a loop B of a CTE<sub>IAP</sub> as defined above; b) contacting the composition with a test compound; and, c) measuring the ability of the test compound to bind the NCTE.

A further understanding of the nature and advantages of the present invention may be realized by reference to the remaining portions of the specification, the figures and claims.

All publications, patents and patent applications cited herein are hereby expressly incorporated by reference for all purposes.

## BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates two RNA sequences: the novel CTE<sub>IAP</sub> of the invention (SEQ ID NO:1, Figure 1A) and SRV-1 CTE (SEQ ID NO:2, Figure 1B). In Figures 1A and 1B the two sequences are shown folded in a secondary structure whose configuration is based on intramolecular base-pairing. This secondary structure includes two loops, A and B, a four nucleotide AAGA "bulge" adjacent to loop A, and a hairpin turn. Loops A (with "bulge") and B are circled.

Figure 2 illustrates how the novel IAP, including the CTE<sub>IAP</sub> of the invention, is inserted into the transcribed osteocalcin related gene (ORG) between its promoter (designated "P") and the ORG coding region, thus displacing ORG's functional promoter by 3.4 kilobases. The Figure shows that the murine osteocalcin gene consists of osteocalcin 1 ("mOG1"), osteocalcin 2 ("mOG2"), and ORG. Exons are designated as Roman numerals; exons Ib and Ic are non-coding exons. The sequence of the ORG promoter is also shown, with the identified start of transcription 47 nucleotides downstream of the ORG TATA box in the 5' LTR (long terminal repeat) of the ORG<sub>IAP</sub>. The location of CTE<sub>IAP</sub> of the invention within the ORG gene, shown as "CTE," and splice



sites are indicated (see Figure 4, where the DNA subsequence encoding CTE<sub>IAP</sub> is between nucleotides 3029-3204 of SEQ ID NO:3).

Figure 3 illustrates data demonstrating that the novel CTE<sub>IAP</sub> is able to replace Rev-mediated activation of HIV-1 in both Jurkat and PBMCs, as shown in Figure 3A and 3B, respectively; and explained in detail in below.

Figure 4 illustrates the nucleic sequence of the novel intracisternal A particle of the invention, ORG<sub>IAP</sub>, which is 3,402 base pairs in length, and flanking imperfect direct repeats (SEQ ID NO:3). The DNA subsequence encoding the CTE<sub>IAP</sub> of the invention is located between nucleotides 3029-3204, as numerated in this figure.

### DETAILED DESCRIPTION OF THE INVENTION

The present invention provides for a novel post-transcriptional RNA nucleo-cytoplasmic transport element (NCTE), designated "CTE<sub>IAP</sub>," derived from a novel intracisternal A particle (IAP). The novel IAP, including the CTE<sub>IAP</sub> of the invention, was found inserted in a murine osteocalcin-related gene (ORG) between its promoter and coding region, and is thus termed "IAP<sub>ORG</sub>."

It was surprisingly found that the novel CTE<sub>IAP</sub> of the invention can be used functionally replace the NCTE of HIV-1, or "RRE." It was discovered that when CTE<sub>IAP</sub> is used in place of RRE to construct an HIV-1 hybrid clone, a slower growing, attenuated virus results. Significantly, while capable of producing infectious virions *in vivo*, this HIV-1 hybrid (a CTE<sub>IAP</sub>-containing RRE-negative recombinant virus) has lower replicative activity than wild-type virus, resulting in an attenuated HIV-1 strain. The level of attenuation can be quantitated using *in vitro* or *in vivo* assays, such as determining the amount of HIV-1 p24<sup>gag</sup> synthesized by hybrid viruses. For example, the CTE<sub>IAP</sub> of the invention, when inserted in an RRE/Rev deficient HIV-1, is capable of functionally reconstituting this post-transcriptional RNA nucleo-cytoplasmic transport system. When the CTE<sub>IAP</sub>-containing hybrid HIV-1 virus infects activated huPBMCs, the level of expression of HIV-1 p24<sup>gag</sup> is between about 50 fold and about 200 fold less than levels of p24<sup>gag</sup> expression when HIV-1 wild type virus, utilizing wild-type NCTE (*i.e.*, RRE), infects activated huPBMCs.

The efficacy of the CTE<sub>IAP</sub>-recombinant hybrid as an attenuating agent in HIV-1 infection and AIDS pathogenesis can be demonstrated using functionally analogous NCTEs, such as the SRV-1 CTE. Recombinant, hybrid HIV-1 clones in which NCTE from SRV-1 (termed "CTE") functionally replaced wild-type HIV-1 NCTE ("RRE") were used to demonstrate the replicative, yet non-cytopathic, effect of CTE-attenuated HIV-1 in the SCID-hu mouse model, as discussed in Example 3. CTE(+)/RRE(-) HIV-1 clones were used to infect Thy/Liv implants (implanted human thymus and liver, see Kollmann (1995) *J. Immunol.* 154:907-921) in SCID-hu mice. Significantly, these viruses propagated slower than both wild-type and Nef-negative HIV-1 clones, demonstrating that they have lower replicative capacity in human lymphocytes. Furthermore, the CTE(+)/RRE(-) attenuated HIV-1 clones were not lymphocytotoxic, no depletion of CD4<sup>+</sup>-bearing cells was observed. This demonstrates that HIV-1 hybrid clones utilizing exogenous NCTEs, as CTE (of SRV) or CTE<sub>IAP</sub> have an attenuated phenotype for cytotoxicity. Analogously, when the CTE<sub>IAP</sub>-attenuated HIV-1 of the invention infects activated human lymphocytes *in vivo*, it will also produce low levels of infectious virions without any lymphocytotoxic effects, *i.e.*, levels of CD4<sup>+</sup> T cells will not decline. Significantly, the invention's CTE<sub>IAP</sub>-containing HIV-1 is significantly more attenuated than SRV-1 CTE-attenuated virus, as indicated by that fact that the level of expression of p24<sup>gag</sup> by CTE<sub>IAP</sub>-containing HIV-1 is between about 50 fold less than wild type virus, while SRV-1 NCTE produces a hybrid virus only about 10 fold less than wild type. Importantly, this CTE<sub>IAP</sub>-attenuated virus will elicit an immune response in the infected, yet asymptomatic, individual.

The finding that an IAP element can be utilized in the attenuation of a retrovirus whose productive infection does not lead to loss of CD4<sup>+</sup> cells is especially unexpected in view of past findings that a human IAP has been found to be associated with CD4<sup>+</sup> T-cell immunodeficiency and dysfunction. The presence of IAP sequence has also been associated with the occurrence of carcinogenesis, as discussed above.

This invention also provides for a vaccine in the form of a pharmacological compositions and a kit. The pharmacological compositions can comprise a pharmaceutically acceptable carrier and the attenuated virus of the invention. The kit can comprise a container containing a vaccine formulation.

**DEFINITIONS**

The term "CTE<sub>IAP</sub>" refers to a family, or genus of nucleic acid sequences defined as having the following structural and functional properties: the CTE<sub>IAP</sub>, when inserted in an HIV-1 is capable of functioning as an NCTE in place of wild-type HIV-1 NCTE (*i.e.*, RRE), and when the CTE<sub>IAP</sub>-containing hybrid HIV-1 virus infects activated huPBMCs, the level of expression of HIV-1 p24<sup>gag</sup> is between about 50 fold to about 200 fold less than levels of p24<sup>gag</sup> expression when HIV-1 wild type virus, utilizing wild-type NCTE, infects activated huPBMCs; and, the secondary structure of the CTE<sub>IAP</sub> comprises at least a two loop A domain and a loop B domain, wherein the nucleic acid primary sequence of the loops has at least 90% nucleic acid sequence identity to a loop A and a loop B domain of a nucleic acid comprising the loop structure as set forth in Figure 1. The nucleic acid of SEQ ID NO:1 is an exemplary specie of the CTE<sub>IAP</sub> family of the invention.

The term "intracisternal A particle" or "IAP" refers to a family of defective endogenous retroviruses found in a variety of mammals. Murine IAPs can have reverse transcriptase activity and can accumulate in the endoplasmic reticulum. IAPs are further described by, *e.g.*, Wilson (1974) *supra*; Leuders (1977) *supra*; Kaushik (1994) *supra*; Kuff (1990) *supra*; Fehrmann (1997) *supra*; and, Wang (1997) *supra*.

The term "IAP<sub>ORG</sub>" refers to the novel intracisternal A particle (IAP) of the invention, including the CTE<sub>IAP</sub> of the invention, which was found inserted in a murine osteocalcin-related gene (ORG) between its promoter and coding region (thus termed "IAP<sub>ORG</sub>").

The term "constitutive transport element" or "RNA nucleocytoplasmic transport element" or "NCTE" refers to *cis*-acting, post-transcriptional RNA nucleocytoplasmic transport elements. Simian retrovirus NCTE is also referred to as "CTE." These RNA sequences typically have a high degree of secondary structure in the form of stem-loop structures, which can interact with *trans*-acting NCTE-binding proteins, hairpin turns, and the like. Some retroviruses, including HIV and simian retroviruses, regulate their growth through expression of their RNA using NCTEs and corresponding NCTE-binding proteins. NCTE binding with viral or cellular *trans*-acting NCTE-binding proteins stabilizes unspliced viral transcripts and allows interaction with cellular machinery to

transport the message from the nucleus to the cytoplasm. HIV-1's NCTE is designated the "Rev-responsive element," or "RRE," contained in its *gag/pol* and *env* encoding transcript. NCTEs are further described, *e.g.*, by Bray (1994) *supra*; Tabernero (1996) *supra*; Zolotukhin (1994) *supra*; Hua (1996) *supra*; Grate (1997) *Structure* 5:7-11.

5           The term "NCTE-binding protein" refers to a *trans*-acting polypeptide which binds to RNA NCTE sequences, typically interacting with specific secondary structures. This RNA binding protein functions with other cellular proteins in the cellular nuclear export machinery to constitutively transport message nucleic acid (mRNA) from the nucleus to the cytoplasm. An NCTE-binding protein can be encoded by endogenous  
10       cellular (eukaryotic) gene or a viral gene. HIV-1 encodes for its own specific, *trans*-acting NCTE-binding polypeptide, termed "Rev." Rev is further described, *e.g.*, Hua (1996) *supra*; Iversen (1995) *supra*.

          The term "HIV" refers to a lentivirus usually called "human immunodeficiency virus" which is believed to be the causal agent of acquired immune  
15       deficiency syndrome, or AIDS. There are several known subtypes of HIV, including HIV-1 and HIV-2. HIV and AIDS are well described in the literature and, *e.g.*, are further described by Gottfredsson (1997) *Front Biosci.* 2: D619-D634; Burton (1997) *Proc. Natl. Acad. Sci. USA* 94:10018-10023; Barnadas (1997) *J. Cutan. Pathol.* 24:507-510; Doms (1997) *Virology* 235:179-190; Cossarizza (1997) *AIDS* 11:1075-1088; Carpenter (1997)  
20       *JAMA* 277:1962-1969; Klein (1995) *Trends Microbiol.* 3:386-391.

          The term "p24<sup>gag</sup> antigen" or "p24<sup>gag</sup>" refers to the 24 kd HIV-1 polypeptide found associated with the virus' RNA genome in the core of the virion, as described, *e.g.*, by Jones (1996) *Nat. Struct. Biol.* 3:818-820 (1996); Doe (1996) *AIDS* 10:793-794; Klenerman (1996) *AIDS* 10:348-350; Klenerman (1994) *Nature* 369:403-407.

25       The term "Nef" refers to a 27 - 34 kD myristoylated protein unique to primate lentiviruses. A functional Nef gene is important for development of high viremia and AIDS. For a description of Nef, see discussion *infra* and, *e.g.*, Saksela (1997) *Front Biosci.* 2: D606-D618; Greenberg (1997) *EMBO J.* 16:6964-6976; Luo (1997) *J. Virol.* 71:9531-9537; Luo (1997) *J. Virol.* 71:9524-9530; Okada (1997) *FEBS Lett.* 417:61-64.

30       The term "capable of functioning as a post-transcriptional RNA nucleocytoplasmic transport element (NCTE) in place of wild-type NCTE in a hybrid HIV-1"

means that the NCTE, such as the claimed CTE<sub>IAP</sub>, when inserted into (is part of the sequence of) an HIV-1 messenger RNA, is capable of interacting with the appropriate *trans*-acting polypeptides to effect the splicing and subsequent transfer of the mRNA from the nucleus to the cytoplasm. An NCTE is still considered "capable of functioning as an NCTE in place of wild-type NCTE in a hybrid HIV-1" even if it is less efficient, less accurate, or less capable, of splicing and/or transferring mRNA (of which it is a part) from the nucleus to the cytoplasm. For example, the CTE<sub>IAP</sub> of the invention, when inserted in HIV-1, is considered capable of functioning as an NCTE in place of wild-type NCTE (RRE) in the hybrid HIV-1 even though it is less efficient than RRE; *i.e.*, when the CTE<sub>IAP</sub>-containing hybrid HIV-1 virus infects activated human peripheral blood mononuclear cells (huPBMCs), the level of expression of HIV-1 p24<sup>gag</sup> is between about 50 fold and about 200 fold less than levels of p24<sup>gag</sup> expression when HIV-1 wild type virus, utilizing wild-type NCTE (RRE), infects activated huPBMCs.

The term "activated" refers to a non-dormant cellular state, for example, as when a lymphocyte has been activated by an antigen, cytokine(s) or other mitogen.

The term "wild-type" refers to any form (*e.g.*, tertiary structure), structure (*e.g.*, secondary structure) or sequence (*e.g.*, primary structure) of a composition, as a nucleic acid or polypeptide, as found in nature, versus structures or sequences which have been manipulated by the hand of man, *i.e.*, recombinant nucleic acids or polypeptides.

The term "secondary structure" refers to the structure formed by the intramolecular folding of a polypeptide or a nucleic acid, such as the stem and loop structures formed by the CTE<sub>IAP</sub> of the invention.

The term "peripheral blood mononuclear cell" refers to any peripheral mononuclear white blood cell.

The term "expression vector" refers to any recombinant expression system for the purpose of expressing a nucleic acid sequence of the invention *in vitro* or *in vivo*, constitutively or inducibly, in any cell, including prokaryotic, yeast, fungal, plant, insect or mammalian cell. The term includes linear or circular expression systems. The term includes expression systems that remain episomal or integrate into the host cell genome.

The expression systems can have the ability to self-replicate or not, *i.e.*, drive only transient

expression in a cell. The term includes recombinant expression "cassettes" which contain only the minimum elements needed for transcription of the recombinant nucleic acid.

The term "isolated," when referring to a molecule or composition, such as, for example, a polypeptide or nucleic acid, means that the molecule or composition is separated from at least one other compound, such as a protein, other nucleic acids (*e.g.*, RNAs), or other contaminants with which it is associated *in vivo* or in its naturally occurring state. Thus, a polypeptide or nucleic acid is considered isolated when it has been isolated from any other component with which it is naturally associated, *e.g.*, cell membrane, as in a cell extract. An isolated composition can, however, also be substantially pure. An isolated composition can be in a homogeneous state and can be in a dry or an aqueous solution. Purity and homogeneity can be determined, for example, using analytical chemistry techniques such as polyacrylamide gel electrophoresis (SDS-PAGE) or high performance liquid chromatography (HPLC).

The term "polynucleotide," "nucleic acid molecule" or "nucleic acid sequence" refers to a deoxyribonucleotide or ribonucleotide oligonucleotide in either single- or double-stranded form. The term encompasses nucleic acids, *i.e.*, oligonucleotides, containing known analogues of natural nucleotides which have similar or improved binding properties, for the purposes desired, as the reference nucleic acid. The term also includes nucleic acids which are metabolized in a manner similar to naturally occurring nucleotides or at rates that are improved thereover for the purposes desired. The term also encompasses nucleic-acid-like structures with synthetic backbones. DNA backbone analogues provided by the invention include phosphodiester, phosphorothioate, phosphorodithioate, methylphosphonate, phosphoramidate, alkyl phosphotriester, sulfamate, 3'-thioacetal, methylene(methylimino), 3'-N-carbamate, morpholino carbamate, and peptide nucleic acids (PNAs, which contain non-ionic backbones, such as N-(2-aminoethyl) glycine units); see *Oligonucleotides and Analogues, A Practical Approach*, edited by F. Eckstein, IRL Press at Oxford University Press (1991); *Antisense Strategies*, Annals of the New York Academy of Sciences, Volume 600, Eds. Baserga and Denhardt (NYAS 1992); Milligan (1993) *J. Med. Chem.* 36:1923-1937; *Antisense Research and Applications* (1993, CRC Press) in its entirety and specifically Chapter 15, by Sanghvi. Phosphorothioate linkages are described in WO 97/03211; WO 96/39154; Mata (1997)

*Toxicol Appl Pharmacol* 144:189-197. Other synthetic backbones encompasses by the term include methylphosphonate linkages or alternating methylphosphonate and phosphodiester linkages (Strauss-Soukup (1997) *Biochemistry* 36:8692-8698), and benzylphosphonate linkages (Samstag (1996) *Antisense Nucleic Acid Drug Dev.*

6:153-156). The term nucleic acid is used interchangeably with gene, DNA, cDNA, RNA, mRNA, oligonucleotide primer, probe and amplification product.

The term "exogenous" as in "exogenous nucleic acid" refers to a molecule (*e.g.*, nucleic acid or polypeptide) that has been isolated, synthesized, and/or cloned, in a manner that is not found in nature, and/or introduced into and/or expressed in a cell or cellular environment other than or at levels or forms different than the cell or cellular environment in which said nucleic acid or protein can be found in nature. The term encompasses both nucleic acids originally obtained from a different organism or cell type than the cell type in which it is expressed, and also nucleic acids that are obtained from the same organism, cell, or cell line as the cell or organism in which it is expressed.

The term "endogenous" refers to a molecule, *e.g.*, a nucleic acid or polypeptide, in a form, structure and/or sequence found in nature.

"Sequence identity" in the context of two nucleic acid or polypeptide sequences includes reference to the nucleotides (or residues) in the two sequences which are the same when aligned for maximum correspondence over a specified "comparison window." Sequence identity analysis is used to determine whether a nucleic acid is within scope of the invention. For example, to identify a specie of the CTE<sub>IAP</sub> family of the invention, a nucleic acid must have a secondary structure with at least a two loop A domain and/or a loop B domain, where the nucleic acid primary sequence of the loops has at least 90% nucleic acid sequence identity to a loop A and a loop B domain of a nucleic acid as set forth in Figure 1, SEQ ID NO:1. "Sequence identity" can be analyzed by optimal alignment of sequences for comparison using any means to analyze sequence identity (homology) known in the art, *e.g.*, by the progressive alignment method of termed "PILEUP"; by the local homology algorithm of Smith & Waterman (1981) *Adv. Appl. Math.* 2: 482; by the homology alignment algorithm of Needleman & Wunsch (1970) *J. Mol. Biol.* 48:443; by the search for similarity method of Pearson (1988) *Proc. Natl. Acad. Sci. USA* 85: 2444; by computerized implementations of these algorithms, *e.g.* BLAST,

GAP, BESTFIT, FASTA, and TFASTA in, *e.g.*, the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI; or, by inspection. See also Morrison (1997) *Mol. Biol. Evol.* 14:428-441, as an example of the use of PileUp, ClustalW, TreeAlign, MALIGN, and SAM sequence alignment computer programs.

5 PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle, *J. Mol. Evol.* 35:351-360 (1987) and is similar to the method described by Higgins & Sharp *CABIOS* 5:151-153 (1989). The BLAST algorithm is described in Altschul (1990) *J. Mol. Biol.* 215: 403-410, and software for performing BLAST analyses is publicly available, *e.g.*, see National Center for Biotechnology Information at <http://www.ncbi.nlm.nih.gov/>.

10 See also Corpet (1988) *Nucleic Acids Res.* 16:10881-90; Huang (1992) *Computer Applications in the Biosciences* 8:155-65; and Pearson (1994) *Methods in Molec. Biol.* 24:307-31.

The term "recombinant," when used with reference to, *e.g.*, a cell, nucleic acid, polypeptide, expression cassette or vector, refers to a material, or a material  
15 corresponding to the natural or native form of the material, that has been modified by the introduction of a new moiety or alteration of an existing moiety, or is identical thereto but produced or derived from synthetic materials. For example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell (*i.e.*, "exogenous nucleic acids") or express native genes that are otherwise expressed at a  
20 different level, typically, under-expressed or not expressed at all. The term "recombinant means" refers to techniques where, *e.g.*, a recombinant nucleic acid such as a cDNA encoding a protein or an antisense sequence, is inserted into an expression cassette, such as an expression vector, the resultant construct is introduced into a cell, and the cell expresses the nucleic acid, and the protein, if appropriate. "Recombinant means" also encompass the  
25 ligation of nucleic acids to coding or promoter sequences from different sources into one expression cassette or vector for expression of a fusion protein, constitutive expression of a protein, or inducible expression of a protein.

The term "test compound" refers to any synthetic or natural compound or composition. The term includes all organic and inorganic compounds; including, for  
30 example, small molecules, peptides, proteins, sugars, nucleic acids, fatty acids and the like.



The term "motif" or "domain" refers to a nucleic acid or amino acid sequence pattern, or structure, which is shared between related molecules.

The term "ameliorating" or "ameliorate" refers to any indicia of success in the treatment of a pathology or condition, including any objective or subjective parameter such as abatement, remission or diminishing of symptoms or an improvement in a patient's physical or mental well-being. Amelioration of symptoms can be based on objective or subjective parameters; including the results of a physical examination and/or a psychiatric evaluation.

The term "prophylaxis" refers to any form of prevention, delay or abatement a pathology or condition or symptom thereof, including any objective or subjective parameter.

The term "attenuated" refers to a state wherein an infectious agent, *i.e.*, a pathogen, such as a microbial or viral agent, has a phenotype manifested by a lessened ability to grow, proliferate, or cause pathogenesis, in a host, *i.e.*, the non-wild type, attenuated phenotype is less virulent. For example, a CTE<sub>IAP</sub>-attenuated HIV-1 virus is capable of replication, infection and production of infectious virions without causing clinically significant pathology in its host.

The term "immune response" in a host refers to both cellular and humoral (antibody) mediated responses to an immunogen, *i.e.*, a compound or composition capable of eliciting an immune response. The immune response can be elicited by a foreign substance or a pathogen, and the immunogen can be a carbohydrate, a nucleic acid, a polypeptide, a lipid, or a combination of these elements.

The term "vaccine" is used in its ordinary sense, meaning an agent which is capable of eliciting a humoral and/or cell-mediated immunoprotective immune response when administered to an individual with an at least partially functioning immune system.

## **I. CHARACTERIZATION AND ISOLATION OF NUCLEIC ACIDS ENCODING IAP and CTE<sub>IAP</sub>**

This invention has for the first time provided for the characterization, cloning and expression of a novel NCTE, CTE<sub>IAP</sub>, derived from a novel intracisternal A particle, IAP<sub>ORG</sub>. The invention also provides for novel means of expressing the IAP<sub>ORG</sub>

and CTE<sub>IAP</sub> of the invention *in vitro* and *in vivo*. In a further embodiment, this expression provides for a means to screen for novel NCTEs.

The invention can be practiced in conjunction with any method or protocol known in the art, which are well described in the scientific and patent literature.

Therefore, only a few general techniques will be described prior to discussing specific methodologies and examples relative to the novel reagents and methods of the invention.

#### A. General Techniques

Methods of isolating total DNA or RNA encoding the nucleic acids of the invention are well known to those of skill in the art. Techniques for isolation, purification and manipulation of nucleic acids, genes and CTE<sub>IAP</sub> sequences, such as generating libraries, subcloning into expression vectors, labeling probes, DNA hybridization, and the like are described, *e.g.*, in Sambrook, MOLECULAR CLONING: A LABORATORY MANUAL (2ND ED.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989) ("Sambrook"); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Ausubel, ed. John Wiley & Sons, Inc., New York (1997) ("Ausubel"); LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY: HYBRIDIZATION WITH NUCLEIC ACID PROBES, *Part I. Theory and Nucleic Acid Preparation*, P. Tijssen, ed. Elsevier, N.Y. (1993) ("Tijssen").

The nucleic acids of this invention, whether RNA, mRNA, DNA, cDNA, genomic DNA, or a hybrid of the genetic recombinations, may be isolated from a variety of sources or may be synthesized *in vitro*. Nucleic acids of the invention can be expressed in transgenic animals, transformed cells, in a transformed cell lysate, or in a partially purified or a substantially pure form. Sequencing methods typically use dideoxy sequencing (Sequenase, U.S. Biochemical), however, other kits and methods are available and well known to those of skill in the art.

Nucleic acids and proteins are detected and quantified in accordance with the teachings and methods of the invention described herein by any of a number of general means well known to those of skill in the art. These include, for example, analytical biochemical methods such as spectrophotometry, radiography, electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), and hyperdiffusion chromatography, various immunological methods, such as fluid or gel precipitin reactions, immunodiffusion (single or double),

immuno-electrophoresis, radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), immuno-fluorescent assays, and the like, Southern analysis, Northern analysis, Dot-blot analysis, gel electrophoresis, RT-PCR, quantitative PCR, other nucleic acid or target or signal amplification methods, radiolabeling, scintillation counting, and affinity chromatography, to name only a few.

**B. Isolation, Synthesis and Purification of Nucleic Acids Encoding IAP<sub>ORG</sub> and CTE<sub>IAP</sub>**

In one embodiment, the invention provides for a novel IAP and CTE<sub>IAP</sub> and methods to identify alleles, isoforms and polymorphisms.

**1. Preparation and Screening of DNA Libraries**

There are numerous methods for isolating the DNA sequences encoding the CTE<sub>IAP</sub> and IAP<sub>ORG</sub> of the invention. For example, DNA can be isolated from a genomic or cDNA library using labeled oligonucleotide probes having sequences complementary to the sequences or subsequences disclosed herein, such as CTE<sub>IAP</sub> and IAP<sub>ORG</sub>, SEQ ID NOS:1 and SEQ ID NO:3, respectively (the DNA subsequence encoding the CTE<sub>IAP</sub> of the invention is located between nucleotides 3029-3204 of SEQ ID NO:3). Such probes can be used directly in hybridization assays to isolate DNA encoding IAP<sub>ORG</sub> or CTE<sub>IAP</sub> isoforms and polymorphisms. Alternatively probes can be designed for use in amplification techniques, such as, *e.g.*, PCR, and nucleic acid encoding IAP<sub>ORG</sub> or CTE<sub>IAP</sub> can be isolated by using methods (see *infra*).

To prepare a cDNA library, mRNA is isolated, reverse transcribed from the mRNA according to procedures well known in the art and inserted into vectors. The vectors are transfected into a recombinant host for propagation, screening and cloning. Methods for making and screening cDNA libraries are well known. See, *e.g.*, Gubler (1983) *Gene* 25:263-269, Sambrook, Ausubel.

To make a genomic library, total DNA is extracted and purified by well-known methods (see, *e.g.*, Sambrook). DNA of appropriate size is produced by known methods, such as mechanical shearing or enzymatic digestion, to yield DNA fragments, *e.g.*, of about 12 to 20 kb. The fragments are then separated, as for example, by gradient centrifugation, or gel electrophoresis, from undesired sizes. Selected fragments can be inserted in bacteriophage or other vectors. These vectors and phage can be packaged *in vitro*, as described, *e.g.*, in Sambrook. Recombinant phage can be analyzed by plaque

hybridization described, *e.g.*, in Benton (1977) *Science* 196:180; Chen (1997) *Methods Mol Biol* 62:199-206. Colony hybridization can be carried out as generally described in, *e.g.*, Grunstein (1975) *Proc. Natl. Acad. Sci. USA* 72:3961-3965; Yoshioka (1997) *J. Immunol Methods* 201:145-155; Palkova (1996) *Biotechniques* 21:982.

5 DNA encoding an IAP or CTE<sub>IAP</sub> can be identified in either cDNA or genomic libraries by hybridization with nucleic acid probes of the invention. For example, a probe containing 10 to 20 to 50 or more contiguous nucleotides of SEQ ID NO:1 or 3 (see Figure 1A and Figure 4) is used in Southern blots to identify CTE<sub>IAP</sub>. Once identified, these DNA regions are isolated by standard methods familiar to those of skill in the art.

10 Alternatively, RNA may be identified by hybridization to nucleic acid probes in Northern blots or other formats; see, *e.g.*, Sambrook, Ausubel, for general procedures.

Oligonucleotides for use as, *e.g.*, probes, templates for further amplification, and the like, can be chemically synthesized, as described below. Synthetic nucleic acids, including oligonucleotide probes and primers, CTE<sub>IAP</sub> and IAP coding sequences, can be prepared by a variety of solution or solid phase methods. Detailed descriptions of the procedures for solid phase synthesis of nucleic acids by phosphite-triester, phosphotriester, and H-phosphonate chemistries are widely available. For example, the solid phase phosphoramidite triester method of Beaucage and Carruthers using an automated synthesizer is described in Itakura, U.S. Pat. No. 4,401,796; Carruthers, U.S. Pat. Nos. 20 4,458,066 and 4,500,707; Carruthers (1982) *Genetic Engineering* 4:1-17. See also Needham-VanDevanter (1984) *Nucleic Acids Res.* 12:6159-6168; Beigelman (1995) *Nucleic Acids Res* 23: 3989-3994; OLIGONUCLEOTIDE SYNTHESIS: A PRACTICAL APPROACH, Gait (ed.), IRL Press, Washington D.C. (1984), see Jones, chapt 2, Atkinson, chapt 3, and Sproat, chapt 4; Froehler (1986) *Tetrahedron Lett.* 27:469-472; Froehler, 25 *Nucleic Acids Res.* 14:5399-5407 (1986); Sinha (1983) *Tetrahedron Lett.* 24:5843-5846; and Sinha (1984) *Nucl. Acids Res.* 12:4539-4557. Methods to purify oligonucleotides include native acrylamide gel electrophoresis, anion-exchange HPLC, as described in Pearson (1983) *J. Chrom.* 255:137-149. The sequence of the synthetic oligonucleotide can be verified using any chemical degradation method, for example, see Maxam (1980) 30 *Methods in Enzymology* 65:499-560, Xiao (1996) *Antisense Nucleic Acid Drug Dev*

6:247-258, or for solid-phase chemical degradation procedures, Rosenthal (1987) *Nucleic Acids Symp Ser* 18:249-252.

## 2. Amplification of Nucleic Acids Encoding CTE<sub>IAP</sub> and IAP

The present invention provides oligonucleotide primers and probes that can  
5 hybridize specifically to nucleic acids having CTE<sub>IAP</sub> and IAP nucleic acid sequences. Such reagents can be used to identify further species, such as polymorphisms alleles and other variations, of CTE<sub>IAP</sub> and IAP. For illustrative purposes, PCR primers and amplification methods are described.

Amplification of CTE<sub>IAP</sub> and IAP sequences which are conserved amongst  
10 different CTE<sub>IAP</sub> and IAP species are preferred reagents of the invention for use as hybridization probes to identify and isolate additional species from various organisms. These oligonucleotides can also be used as primers to directly amplify nucleic acids sequences, as described below.

Oligonucleotides can be used to identify and detect additional CTE<sub>IAP</sub>  
15 species using a variety of hybridization techniques and conditions. Suitable amplification methods include, but are not limited to: polymerase chain reaction, PCR (PCR PROTOCOLS, A GUIDE TO METHODS AND APPLICATIONS, *ed.* Innis, Academic Press, N.Y. (1990) and PCR STRATEGIES (1995), *ed.* Innis, Academic Press, Inc., N.Y. (Innis )), ligase chain reaction (LCR) (Wu (1989) *Genomics* 4:560; Landegren (1988) *Science*  
20 241:1077; Barringer (1990) *Gene* 89:117); transcription amplification (Kwoh (1989) *Proc. Natl. Acad. Sci. USA* 86:1173); and, self-sustained sequence replication (Guatelli (1990) *Proc. Natl. Acad. Sci. USA*, 87:1874); Q Beta replicase amplification and other RNA polymerase mediated techniques (*e.g.*, NASBA, Cangene, Mississauga, Ontario); see  
25 Berger (1987) *Methods Enzymol.* 152:307-316, Sambrook, and Ausubel, as well as Mullis (1987) U.S. Patent Nos. 4,683,195 and 4,683,202; Arnheim (1990) *C&EN* 36-47; Lomell *J. Clin. Chem.*, 35:1826 (1989); Van Brunt, *Biotechnology*, 8:291-294 (1990); Wu (1989) *Gene* 4:560; Sooknanan (1995) *Biotechnology* 13:563-564. Methods for cloning *in vitro* amplified nucleic acids are described in Wallace, U.S. Pat. No. 5,426,039.

The invention provides for amplification and manipulation or detection of  
30 the products from each of the above methods to prepare DNA encoding CTE<sub>IAP</sub> and IAP nucleic acid. In PCR techniques, oligonucleotide primers complementary to the two

borders of the DNA region to be amplified are synthesized and used (see, Innis). PCR can be used in a variety of protocols to amplify, identify, quantify, isolate and manipulate nucleic acids encoding CTE<sub>IAP</sub> and IAP. In these protocols, primers and probes for amplification and hybridization are generated that comprise all or any portion of the DNA sequences listed herein

An illustrative primer pair that can amplify the CTE<sub>IAP</sub> of the invention under appropriate conditions is:

sense: 5'-CAACTGTTTCTTGGCATGCTAGAGAAGTAGT-3' (SEQ ID NO:4); and antisense 5'-CTGTCTTTACTTATTGGCAGAGAGAA-3' (SEQ ID NO:5).

PCR-amplified sequences can also be labeled and used as detectable oligonucleotide probes, but such nucleic acid probes can be generated using any synthetic or other technique well known in the art, as described above. The labeled amplified DNA or other oligonucleotide or nucleic acid of the invention can be used as probes to further identify and isolate CTE<sub>IAP</sub> and IAP species from various cDNA or genomic libraries.

Another useful means of obtaining nucleic acids of the invention, such as large genomic clones, is to screen YAC, BAC or P1 genomic libraries. BACs, bacterial artificial chromosomes, are vectors that can contain 120+ Kb inserts. BACs are based on the *E. coli* F factor plasmid system and simple to manipulate and purify in microgram quantities. Because BAC plasmids are kept at one to two copies per cell, the problems of rearrangement observed with YACs, which can also be employed in the present methods, are eliminated. BAC vectors can include marker genes for luciferase and green fluorescent protein (GFP). (Baker (1997) *Nucleic Acids Res* 25:1950-1956). Yeast artificial chromosomes, or YACS, can also be used for contain inserts ranging in size from 80 to 700 kb, see, *e.g.*, Tucker (1997) *Gene* 199:25-30; Adam (1997) *Plant J.* 11:1349-1358. P1 is a bacteriophage that infects *E. coli* that can contain 75-100 Kb DNA inserts (Mejia (1997) *Genome Res* 7:179-186; Ioannou (1994) *Nat Genet* 6:84-89), and are screened in much the same way as lambda libraries.

### 3. Cloning of CTE<sub>IAP</sub>- and IAP-Encoding Inserts

The invention also provides CTE<sub>IAP</sub>- and IAP-encoding expression vectors to produce large quantities of full or partial length (*e.g.*, a single RNA loop structure) nucleic acid molecules of the invention. The expression vector, including expression cassettes, include, *e.g.*, those used in bacterial, yeast, plant, insect, *in vitro*, or mammalian systems. For example, generation of CTE<sub>IAP</sub> in this manner is useful for assaying for CTE<sub>IAP</sub> activity modulators, analysis of the activity of newly isolated species of CTE<sub>IAP</sub>, identifying and isolating compounds which specifically associate with CTE<sub>IAP</sub>, such as binding proteins, analysis of the activity of CTE<sub>IAP</sub> which has been site-specifically mutated, as described below. Nucleic acid so produce can be used the the invention's method to screening for a NCTE binding proteins using a single loop A or a loop B, or both loops as in Figure 1A, of a CTE<sub>IAP</sub> of the invention. The nucleic acids of the invention can also be used as immunogens, as a few examples. See, *e.g.*, Radic (1994) *Annu. Rev. Immunol.* 12:487-520; Cabral (1997) *Curr. Opin. Rheumatol.* 9:387-392; Pisetsky (1997) *Methods* 11:55-61; Marion (1997) *Methods* 11:3-11, for general discussion on anti-DNA antibodies; for discussion on generation of anti-RNA antibodies using combinatorial phage display libraries see Marchbank (1995) *Nucleic Acids Symp. Ser.* 33:120-122.

There are several well-known methods of introducing nucleic acids into bacterial and other cells, a process often called "transforming," any of which may be used in the methods of the present invention (see, *e.g.*, Sambrook). Techniques for transforming a wide variety of animal and plant cells are well known and described in the technical and scientific literature. See, *e.g.*, Weising (1988) *Ann. Rev. Genet.* 22:421-477, for plant cells and Sambrook for animal and bacterial cells.

### 4. Sequencing of CTE<sub>IAP</sub>- and IAP-Encoding Nucleic Acid

Sequencing of newly isolated DNA will identify and characterize CTE<sub>IAP</sub>- and IAP-encoding nucleic acid of the invention. Sequencing of isolated CTE<sub>IAP</sub>- and IAP-encoding nucleic acid can be used to identify, in addition to functional criteria, new CTE<sub>IAP</sub>- and IAP-encoding species or allelic variations. Secondary structures can be identified. For example, sequencing of CTE<sub>IAP</sub> and subsequent computer predicted analysis of its secondary structure revealed a loop A with a "bulge" (*e.g.*, an "AAGA")

bulge), a loop B, a stem structure separating the loops and a hairpin turn, see Figure 1, SEQ ID NO:1. In terms of primary sequence criteria, a nucleic acid is a CTE<sub>IAP</sub> specie within the scope of the claimed invention if the sequence of its loop domains has at least 90% nucleic acid sequence identity to the loop A and loop B domains of CTE<sub>IAP</sub> illustrated in Figure 1, SEQ ID NO:1 (the DNA subsequence encoding CTE<sub>IAP</sub> is located between nucleotides 3029-3204 of Figure 4, SEQ ID NO:3).

CTE<sub>IAP</sub>-encoding nucleic acid sequences can be sequenced as inserts in vectors, as inserts released and isolated from the vectors or in any of a variety of other forms (*i.e.*, as amplification products). CTE<sub>IAP</sub>- and IAP-encoding inserts can be released from the vectors by restriction enzymes or amplified by PCR or transcribed by a polymerase. For sequencing of the inserts to identify full length CTE<sub>IAP</sub> or IAP coding sequences, primers based on the N- or C- terminus, or based on insertion points in the original phage or other vector, can be used. Additional primers can be synthesized to provide overlapping sequences.

A variety of nucleic acid sequencing techniques are well known and described in the scientific and patent literature, *e.g.*, see Rosenthal (1987) *supra*; Arlinghaus (1997) *Anal. Chem.* 69:3747-3753, for use of biosensor chips for sequencing; Pastinen (1996) *Clin. Chem.* 42:1391-1397; Nyren (1993) *Anal Biochem.* 208:171-175.

### 5. Nucleic Acid Hybridization Techniques

The hybridization techniques disclosed herein can be utilized to identify, isolate and characterize genes and gene products (*i.e.*, mRNA) encoding IAP and CTE<sub>IAP</sub>-encoding nucleic acid of the invention, including different species, isoforms, alleles and polymorphisms. A variety of methods for specific DNA and RNA measurement using nucleic acid hybridization techniques are known to those of skill in the art. *See. e.g.*, NUCLEIC ACID HYBRIDIZATION, A PRACTICAL APPROACH, Ed. Hames, B.D. and Higgins, S.J., IRL Press, 1985; Sambrook..

One method for evaluating the presence or absence of DNA encoding IAP or CTE<sub>IAP</sub> in a sample involves a Southern transfer. Briefly, the digested bacterial genomic DNA is run on agarose slab gels in buffer and transferred to membranes. Hybridization is carried out using nucleic acid probes. The nucleic acid probes can be designed based on conserved nucleic acid sequences amongst the class of proteins.



Preferably nucleic acid probes are 20 bases or longer in length (see, *e.g.*, Sambrook for methods of selecting nucleic acid probe sequences for use in nucleic acid hybridization). Visualization of the hybridized portions allows the qualitative determination of the presence or absence of DNA encoding IAP or CTE<sub>IAP</sub>.

5 Similarly, a Northern transfer can be used for the detection of RNA containing IAP or CTE<sub>IAP</sub> sequences. For example, RNA is isolated from a given cell sample using an acid guanidinium-phenol-chloroform extraction method. The RNA is then electrophoresed to separate different species and transferred from the gel to a nitrocellulose membrane. As with the Southern transfers, labeled probes or PCR can be  
10 used to identify the presence or absence of IAP or CTE<sub>IAP</sub> nucleic acid.

Sandwich assays are commercially useful hybridization assays for detecting or isolating protein or nucleic acid. Such assays utilize a "capture" nucleic acid or protein that is often covalently immobilized to a solid support and a labeled "signal" nucleic acid, typically in solution. A clinical or other sample provides the target nucleic acid or protein.  
15 The "capture" nucleic acid or protein and "signal" nucleic acid or protein hybridize with or bind to the target nucleic acid or protein to form a "sandwich" hybridization complex. To be effective, the signal nucleic acid or protein cannot hybridize or bind substantially with the capture nucleic acid or protein.

Typically, oligonucleotide probes are labeled signal nucleic acids that are  
20 used to detect hybridization. Complementary probe nucleic acids or signal nucleic acids may be labeled by any one of several methods typically used to detect the presence of hybridized polynucleotides. Methods of detection can use labels for autoradiography or autofluorography, such as <sup>3</sup>H, <sup>125</sup>I, <sup>35</sup>S, <sup>14</sup>C, or <sup>32</sup>P-labeled probes or the like (see definition of label, above). Other labels include ligands which bind to labeled antibodies,  
25 fluorophores, chemiluminescent agents, enzymes, and antibodies which can serve as specific binding pair members for a labeled ligand.

Detection of a hybridization complex may require the binding of a signal generating complex to a duplex of target and probe polynucleotides or nucleic acids. Typically, such binding occurs through ligand and anti-ligand interactions as between a  
30 ligand-conjugated probe and an anti-ligand conjugated with a signal, *i.e.*, antibody-antigen or complementary nucleic acid binding. The label may also allow indirect detection of the

hybridization complex. For example, where the label is a hapten or antigen, the sample can be detected by using antibodies. In these systems, a signal is generated by attaching fluorescent or enzymatic molecules to the antibodies or, in some cases, by attachment of a radioactive label. The sensitivity of the hybridization assays may be enhanced through use of a target nucleic acid or signal amplification system which multiplies the target nucleic acid or signal being detected. *In vitro* amplification techniques suitable for amplifying sequences for use as molecular probes or for generating nucleic acid fragments for subsequent subcloning are known, as described above. These systems can be used to directly identify IAP or CTE<sub>IAP</sub> variations, polymorphisms, or mutated sequences where the PCR or LCR primers or other reagents are designed to be extended or ligated only when a specific sequence is present. Alternatively, the specific sequences can be generally amplified using, for example, more generic PCR primers and the amplified target region later probed or sequenced to identify a specific sequence indicative of the variant, polymorphism or mutation.

Nucleic acid hybridization assays for the detection of isoforms, mutations and for sequencing can also be performed in an array-based format. Arrays are a multiplicity of different "probe" or "target" nucleic acids (or other compounds) are hybridized against a target nucleic acid. In this manner a large number of different hybridization reactions can be run essentially "in parallel". This provides rapid, essentially simultaneous, evaluation of a wide number of reactants. Methods of performing hybridization reactions for detection and sequencing in array based formats are well known to those of skill in the art, *e.g.*, Pastinen (1997) *Genome Res.* 7:606-614; (1997) Jackson (1996) *Nature Biotechnology* 14:1685; Chee (1995) *Science* 274:610.

An alternative means for determining the level of expression of a gene is *in situ* hybridization. *In situ* hybridization assays are well known (*e.g.*, Angerer (1987) *Methods Enzymol* 152:649). In an *in situ* hybridization assay, cells are fixed to a solid support, typically a glass slide. If a nucleic acid is to be probed, the cells are typically denatured with heat or alkali. The cells are then contacted with a hybridization solution at a moderate temperature to permit annealing of labeled probes specific to the nucleic acid sequence encoding the protein. The probes are typically labeled, *i.e.*, with radioisotopes or fluorescent reporters. Another well-known *in situ* hybridization technique is the so-called

FISH fluorescence *in situ* hybridization, as described by Macechko (1997) *J. Histochem. Cytochem.* 45:359-363; and, Raap (1995) *Hum. Mol. Genet.* 4:529-534.

The sensitivity of the hybridization assays may be enhanced through use of a nucleic acid amplification system which multiplies the target nucleic acid being detected. *In vitro* amplification techniques suitable for amplifying sequences for use as molecular probes or for generating nucleic acid fragments for subsequent subcloning are known, as described above. Nucleic acid sequence-based amplifications are also used to directly identify mutants where the PCR or LCR primers are designed to be extended or ligated only when a select sequence is present. Alternatively, the select sequences can be generally amplified using nonspecific PCR primers and the amplified target region later probed for a specific sequence indicative of a mutation.

Oligonucleotides for use as probes, *e.g.*, *in vitro* amplification methods, as gene probes in diagnostic methods, or as inhibitor components (see below) are typically synthesized chemically; *e.g.*, such as by the solid phase phosphoramidite triester method described by Beaucage and Caruthers, *supra*, or, using an automated synthesizer, as described in Needham-VanDevanter, *supra*. Purification of oligonucleotides, where necessary, is typically performed by native acrylamide gel electrophoresis or by anion-exchange HPLC as described in Pearson and Regnier. The sequence of the synthetic oligonucleotides can be verified using the chemical degradation method of Maxam and Gilbert (Maxam (1980) *supra*).

It will be appreciated that nucleic acid hybridization assays can also be performed in an array-based format. In this approach, arrays bearing a multiplicity of different "probe" nucleic acids are hybridized against a target nucleic acid. In this manner a large number of different hybridization reactions can be run essentially "in parallel". This provides rapid, essentially simultaneous, evaluation of a wide number of reactants. Methods of performing hybridization reactions in array based formats are well known to those of skill in the art (*see, e.g.*, Jackson (1996) *Nature Biotechnol.* 14:1685, and Chee (1995) *Science* 274:610).

#### 6. Sequence Comparison Analysis of the CTE<sub>IAP</sub> and IAP<sub>ORG</sub>

IAP and NCTE-encoding nucleic acid sequences of the invention include both genes (genomic sequences, including introns and 5' and 3' non-coding regions) and

gene transcription products (mRNA) identified and characterized by analysis of IAP<sub>ORG</sub> and CTE<sub>IAP</sub> sequences. Optimal alignment of sequences for comparison can be conducted as described above (see definitions).

Sequence identity analysis, in addition to functional analysis, is used to determine whether a nucleic acid is within scope of the invention. For example, in one embodiment, a CTE<sub>IAP</sub> sequence of the invention has a secondary structure with at least a loop A domain, a stem structure separating the loops, and a loop B domain, where the nucleic acid primary sequence of the loops has at least 90% nucleic acid sequence identity to a loop A and a loop B domain of a nucleic acid as set forth in Figure 1 and the sequence as set forth in SEQ ID NO:1.

## II. MUTAGENESIS OF CTE<sub>IAP</sub> and IAP<sub>ORG</sub> NUCLEIC ACID

The invention also provides for CTE<sub>IAP</sub> and IAP<sub>ORG</sub> that have been modified in a site-specific manner to modify, add to, or delete some or all of the nucleic acid's functions. For example, specific base pairs can be modified to alter, increase or decrease the affinity of binding proteins, thus modifying the relative level of attenuation of the CTE; or, modifications can change the stability of the secondary structure of the nucleic acid; or, base pair changes can augment expression of the nucleic acid in a cell, such as a bacteria; as a few illustrative examples.

Site-specific mutations can be introduced into CTE<sub>IAP</sub> and IAP<sub>ORG</sub>-encoding nucleic acid by a variety of conventional techniques, well described in the scientific and patent literature. Illustrative examples include: site-directed mutagenesis by overlap extension polymerase chain reaction (OE-PCR), as in Urban (1997) *Nucleic Acids Res.* 25:2227-2228; Ke (1997) *Nucleic Acids Res* 25:3371-3372, and Chattopadhyay (1997) *Biotechniques* 22:1054-1056, describing PCR-based site-directed mutagenesis "megaprimer" method; Bohnsack (1997) *Mol. Biotechnol.* 7:181-188; Ailenberg (1997) *Biotechniques* 22:624-626, describing site-directed mutagenesis using a PCR-based staggered re-annealing method without restriction enzymes; Nicolas (1997) *Biotechniques* 22:430-434, site-directed mutagenesis using long primer-unique site elimination and exonuclease III.

Modified CTE<sub>IAP</sub> and IAP<sub>ORG</sub> of the invention can be further produced by chemical modification methods, see, e.g., Belousov (1997) *Nucleic Acids Res.* 25:3440-3444; Frenkel (1995) *Free Radic. Biol. Med.* 19:373-380; Blommers (1994) *Biochemistry* 33:7886-7896.

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### III. EXPRESSION OF CTE<sub>IAP</sub> and IAP<sub>ORG</sub> NUCLEIC ACID

The invention provides for methods and reagents the expression of novel CTE<sub>IAP</sub> and IAP<sub>ORG</sub> of the invention in any prokaryotic, eukaryotic, yeast, fungal, plant, insect, human or animal cell. Antisense, in addition to sense, sequences are provided for by the invention. To create cell-based and *in vitro* assay systems to screen for novel NCTEs using CTE<sub>IAP</sub>, a variety of *in vivo* and *in vitro* expression systems are provided by the invention.

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#### A. Vectors and Transcriptional Control Elements

The invention provides for methods and reagents for expressing the novel CTE<sub>IAP</sub> and IAP<sub>ORG</sub> nucleic acids described above as sense or antisense coding sequences. Other embodiments of the invention provide methods and reagents for identifying, isolating and using CTE<sub>IAP</sub> and IAP<sub>ORG</sub> *cis*-acting transcriptional control elements and *trans*-acting NCTE binding proteins. After the coding region of a CTE<sub>IAP</sub> and IAP<sub>ORG</sub> gene has been identified, the expression of natural or synthetic CTE<sub>IAP</sub> and IAP<sub>ORG</sub>-encoding or other (*i.e.*, antisense, ribozyme) nucleic acids can be achieved by operably linking the coding region to transcriptional regulatory elements, such as promoters and enhancers. These sequences have characteristic subsequences, for instance, promoter sequence elements typically include the TATA box consensus sequence (TATAAT), which is usually 20 to 30 base pairs upstream of the transcription start site. Promoters can be tissue-specific or not, constitutive or inducible. Promoters that drive expression continuously under physiological conditions are referred to herein as "constitutive" promoters and are active under most environmental conditions and states of development or cell differentiation. Typical expression systems, such as expression cassettes and vectors, also contain transcription and translation terminators, transcription and translation initiation sequences. Generic expression cassettes typically contain at least one independent terminator sequence, sequences permitting replication of the cassette in eukaryotes, or

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prokaryotes, or both, (*e.g.*, shuttle vectors) and selection markers for both prokaryotic and eukaryotic systems. *See, e.g.*, Roberts (1987) *Nature* 328:731; Berger (1987) *supra*; Schneider (1995) *Protein Expr. Purif.* 6435:10; Sambrook and Ausubel. Product information from manufacturers of biological reagents and experimental equipment also provide information regarding known biological methods. Such manufacturers include the SIGMA chemical company (Saint Louis, MO), R&D systems (Minneapolis, MN), Pharmacia Biotech (Piscataway, NJ), Clontech Laboratories, Inc. (Palo Alto, CA), Aldrich Chemical Company (Milwaukee, WI), GIBCO BRL Life Technologies, Inc. (Gaithersburg, MD), Fluka Chemica-Biochemika Analytika (Fluka Chemie AG, Buchs, Switzerland), Applied Biosystems (Foster City, CA), as well as many other commercial sources known to one of skill. The promoters and vectors used in this invention can be isolated from natural sources, obtained from such sources as ATCC or GenBank libraries, or prepared by synthetic methods, as described herein.

The CTE<sub>IAP</sub> and IAP<sub>ORG</sub> sense or antisense sequences of the invention can be expressed in vectors which are transiently expressed in cells using, *e.g.*, episomal vectors such as vaccinia virus, see Cooper (1997) *Proc Natl Acad Sci U S A* 94:6450-6455. Alternatively, CTE<sub>IAP</sub> and IAP<sub>ORG</sub> coding sequences can be inserted into the host cell genome becoming an integral part of the host chromosomal DNA, using for example, retroviral vectors such as SIV or HIV, see for example, Naldini (1996) *Science* 272:263-267. Expression vectors can contain selection markers that confer a selectable phenotype on transformed cells and sequences coding for episomal maintenance and replication such that integration into the host genome is not required. For example, the marker may encode antibiotic resistance, particularly resistance to chloramphenicol, kanamycin, G418, bleomycin and hygromycin, to permit selection of those cells transformed with the desired DNA sequences, see for example, Blondelet-Rouault (1997) *Gene* 190:315-317. Because selectable marker genes conferring resistance to substrates like neomycin or hygromycin can only be utilized in tissue culture, chemoresistance genes are also used as selectable markers *in vitro* and *in vivo*. Various target cells are rendered resistant to anticancer drugs by transfer of chemoresistance genes encoding, *e.g.*, P-glycoprotein, multidrug resistance-associated protein-transporter, dihydrofolate reductase, glutathione -S-transferase, O 6-alkylguanine DNA alkyltransferase, or aldehyde

reductase (Licht (1997) *Stem Cells* 15:104-111) and the like. Illustrative vectors incorporating CTE<sub>IAP</sub> and IAP<sub>ORG</sub> genes include, for example, adenovirus-based vectors (Cantwell (1996) *Blood* 88:4676-4683; Ohashi (1997) *Proc Natl Acad Sci USA* 94:1287-1292), Epstein-Barr virus-based vectors (Mazda (1997) *J Immunol Methods* 204:143-151), adenovirus-associated virus vectors, Sindbis virus vectors (Strong (1997) *Gene Ther* 4: 624-627), Herpes simplex virus vectors (Kennedy (1997) *Brain* 120: 1245-1259) and retroviral vectors (Schubert (1997) *Curr Eye Res* 16:656-662). Epstein-Barr virus episomal vectors (Horlick (1997) *Protein Expr. Purif.* 9:301-308, and plasmid DNA (Lowrie (1997) *Vaccine* 15: 834-838); all of which can be used to express the nucleic acids of the invention *in vivo* or *ex vivo*

#### IV. INHIBITING EXPRESSION OF CTE<sub>IAP</sub> and IAP<sub>ORG</sub> NUCLEIC ACID

The invention further provides for nucleic acids complementary to, *i.e.*, antisense sequences to, the CTE<sub>IAP</sub> and IAP<sub>ORG</sub> of the invention. Antisense sequences are capable of inhibiting the transport, splicing or transcription of CTE<sub>IAP</sub> and IAP<sub>ORG</sub>-encoding genes. The inhibition can be effected through the targeting of genomic DNA or messenger RNA. The transcription or function of targeted nucleic acid can be inhibited, for example, by hybridization and/or cleavage. One particularly useful set of inhibitors provided by the present invention includes oligonucleotides which are able to either bind CTE<sub>IAP</sub> and IAP<sub>ORG</sub> gene or message, in either case preventing or inhibiting the production or function of CTE<sub>IAP</sub> and IAP<sub>ORG</sub>. The association can be though sequence specific hybridization. Such inhibitory nucleic acid sequences can, for example, be used to completely inhibit or further depress the replicative potential of an attenuated virus. Another useful class of inhibitors includes oligonucleotides which cause inactivation or cleavage of CTE<sub>IAP</sub> and IAP<sub>ORG</sub> message. The oligonucleotide can have enzyme activity which causes such cleavage, such as ribozymes. The oligonucleotide can be chemically modified or conjugated to an enzyme or composition capable of cleaving the complementary nucleic acid. One may screen a pool of many different such oligonucleotides for those with the desired activity.

## 1. Antisense Oligonucleotides

The invention provides for with antisense oligonucleotides capable of binding CTE<sub>IAP</sub> and IAP<sub>ORG</sub> message which can inhibit CTE<sub>IAP</sub> and IAP<sub>ORG</sub> activity by targeting mRNA. Strategies for designing antisense oligonucleotides are well described in the scientific and patent literature, and the skilled artisan can design such CTE<sub>IAP</sub> and IAP<sub>ORG</sub> oligonucleotides using the novel reagents of the invention. In some situations, naturally occurring nucleic acids used as antisense oligonucleotides may need to be relatively long (18 to 40 nucleotides) and present at high concentrations. A wide variety of synthetic, non-naturally occurring nucleotide and nucleic acid analogues are known which can address this potential problem. For example, peptide nucleic acids (PNAs) containing non-ionic backbones, such as N-(2-aminoethyl) glycine units can be used. Antisense oligonucleotides having phosphorothioate linkages can also be used, as described in WO 97/03211; WO 96/39154; Mata (1997) *Toxicol Appl Pharmacol* 144:189-197; Antisense Therapeutics, ed. Agrawal (Humana Press, Totowa, N.J., 1996). Antisense oligonucleotides having synthetic DNA backbone analogues provided by the invention can also include phosphoro-dithioate, methylphosphonate, phosphoramidate, alkyl phosphotriester, sulfamate, 3'-thioacetal, methylene(methylimino), 3'-N-carbamate, and morpholino carbamate nucleic acids, as described above.

Combinatorial chemistry methodology can be used to create vast numbers of oligonucleotides that can be rapidly screened for specific oligonucleotides that have appropriate binding affinities and specificities toward any target, such as the sense and antisense CTE<sub>IAP</sub> and IAP<sub>ORG</sub> sequences of the invention (for general background information, see, e.g., Gold (1995) *J. of Biol. Chem.* 270:13581-13584).

## 2. Inhibitory Ribozymes

The invention provides for with ribozymes capable of binding CTE<sub>IAP</sub> and IAP<sub>ORG</sub> message which can inhibit CTE<sub>IAP</sub> and IAP<sub>ORG</sub> activity by targeting mRNA. Strategies for designing ribozymes and selecting the CTE<sub>IAP</sub> and IAP<sub>ORG</sub>-specific antisense sequence for targeting are well described in the scientific and patent literature, and the skilled artisan can design such CTE<sub>IAP</sub> and IAP<sub>ORG</sub> ribozymes using the novel reagents of the invention. Ribozymes act by binding to a target RNA through the target RNA binding portion of a ribozyme which is held in close proximity to an enzymatic portion of the RNA



that cleaves the target RNA. Thus, the ribozyme recognizes and binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cleave and inactivate the target RNA. Cleavage of a target RNA in such a manner will destroy its ability to direct synthesis of an encoded protein if the cleavage occurs in the coding sequence, or, as with CTE<sub>LAP</sub>, prevent viral message from binding to NCTE binding protein, thus preventing subsequent transport of the message to the cytoplasm. After a ribozyme has bound and cleaved its RNA target, it is typically released from that RNA and so can bind and cleave new targets repeatedly.

In some circumstances, the enzymatic nature of a ribozyme can be advantageous over other technologies, such as antisense technology (where a nucleic acid molecule simply binds to a nucleic acid target to block its transcription, translation or association with another molecule, as with CTE<sub>LAP</sub>) as the effective concentration of ribozyme necessary to effect a therapeutic treatment can be lower than that of an antisense oligonucleotide. This potential advantage reflects the ability of the ribozyme to act enzymatically. Thus, a single ribozyme molecule is able to cleave many molecules of target RNA. In addition, a ribozyme is typically a highly specific inhibitor, with the specificity of inhibition depending not only on the base pairing mechanism of binding, but also on the mechanism by which the molecule inhibits the expression of the RNA to which it binds. That is, the inhibition is caused by cleavage of the RNA target and so specificity is defined as the ratio of the rate of cleavage of the targeted RNA over the rate of cleavage of non-targeted RNA. This cleavage mechanism is dependent upon factors additional to those involved in base pairing. Thus, the specificity of action of a ribozyme can be greater than that of antisense oligonucleotide binding the same RNA site.

The enzymatic ribozyme RNA molecule can be formed in a hammerhead motif, but may also be formed in the motif of a hairpin, hepatitis delta virus, group I intron or RNaseP-like RNA (in association with an RNA guide sequence). Examples of such hammerhead motifs are described by Rossi (1992) *Aids Research and Human Retroviruses* 8:183; hairpin motifs by Hampel (1989) *Biochemistry* 28:4929, and Hampel (1990) *Nuc. Acids Res.* 18:299; the hepatitis delta virus motif by Perrotta (1992) *Biochemistry* 31:16; the RNaseP motif by Guerrier-Takada (1983) *Cell* 35:849; and the group I intron by Cech U.S. Pat. No. 4,987,071. The recitation of these specific motifs is not intended to be

limiting; those skilled in the art will recognize that an enzymatic RNA molecule of this invention has a specific substrate binding site complementary to one or more of the target gene RNA regions, and has nucleotide sequence within or surrounding that substrate binding site which imparts an RNA cleaving activity to the molecule.

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## V. CONSTRUCTION OF ATTENUATED VIRUS AND VIRAL VACCINE

The invention provides for an attenuated retrovirus and vaccine comprising the CTE<sub>IAP</sub> of the invention. One means to genetically engineer a wild-type, virulent virus to a hybrid, attenuated virus involves constructing a virus which either lacks or has a non-functional endogenous post-transcriptional RNA nucleo-cytoplasmic transport elements (NCTEs). The endogenous NCTE is subsequently replaced by the exogenous NCTE of the invention which functions less efficiently *in vivo* than its wild-type counterpart, thus effecting the attenuation. The CTE<sub>IAP</sub> of the invention, when inserted in a RRE(-) and/or Rev(-) HIV-1, is capable of functioning as a less efficient, "attenuated" NCTE in place of the wild-type RRE/Rev NCTE system. This level of attenuation can be measured; when the CTE<sub>IAP</sub>-containing hybrid HIV-1 virus infects activated huPBMCs, the level of expression of HIV-1 p24<sup>gag</sup> is between about 50 fold and about 200 fold less than levels of p24<sup>gag</sup> expression when HIV-1 wild type virus, utilizing wild-type NCTE, infects activated huPBMCs. Furthermore, in constructing the attenuated retrovirus of the invention, additional elements of the retrovirus which are essential for its replication and/or pathogenicity can also be disabled or eliminated, such as Nef, as explained below.

In normal mammalian cells, message RNA, present in the cell as ribonucleoprotein (RNP) complexes, is only exported from the nucleus to the cytoplasm after splicing is completed. To circumvent the requirement of splicing prior to export from the nucleus, all retroviruses have evolved a mechanism that allows the nuclear export of unspliced form of viral RNAs which are necessary for the production of structural proteins and essential for viral replication. This mechanism involves the highly structured NCTE *cis*-acting RNA element and its corresponding *trans*-acting RNA binding proteins, as discussed above. In simian type retroviruses, the NCTE is termed "CTE" (see Bray (1994) *supra*; Zolotukhin (1994) *supra*), and binds to endogenous cellular RNA binding proteins. In contrast, HIV-1's NCTE does not bind cellular NCTE-binding proteins, it encodes its

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own NCTE binding protein, called "Rev." Rev interacts with a specific HIV-1 NCTE sequence, designated the "Rev-responsive element," or "RRE," contained in its *gag/pol* and *env* encoding transcript. Rev interacts directly with RRE as part of the RNA export machinery which transports RRE-containing transcripts to the cytoplasm from the nucleus. As a result, HIV-1 needs both RRE and Rev to produce infectious virus. Disabling either produces a non-replicative, non-virulent virus. Replacing (*i.e.*, reconstituting) HIV-1's RRE/Rev RNA transport mechanism with a less efficient NCTE, such as the CTE<sub>IAP</sub> of the invention, produces an attenuated, avirulent hybrid virus.

To engineer a non-functional RRE and/or Rev, the skilled artisan can delete and or mutate any portion of the RRE or Rev coding sequence. Means to delete or mutate nucleic acid sequence are described herein, and are well known in the art. Construction of exemplary, attenuated retroviruses are also discussed in the Examples, below. RRE and Rev sequences are well known in the art, *e.g.*, see databases, such as the NCBI database at <http://www.ncbi.nlm.nih.gov/Entrez/nucleotide.html> or <http://www.ncbi.nlm.nih.gov/Entrez/protein.html>. Further description and sequence of HIV-1 Rev can be found in, *e.g.*, Salminen (1997) *J. Virol.* 71:2647-2655, Accession U86770; Theodore (1996) *AIDS Res. Hum. Retroviruses* 12:191-194, Accession AF004394; Fang, *et al.*, Accession AF003887; Howard (1996) *AIDS Res. Hum. Retroviruses* 12:1413-1425, Accession L39106; to name only a few. Further description and sequence of HIV-1 RRE can be found in, *e.g.*, Salminen (1996) *JOURNAL AIDS Res. Hum. Retroviruses* 12:1329-1339, Accession U46016; WO 9202228-A5 20-FEB-1992, Accession A20711; Battiste (1994) *Biochemistry* 33:2741-2747; Battiste (1995) *J. Biomol. NMR* 6:375-389; Battiste (1996) *Science* 273:1547-1551; to name just a few.

In constructing the attenuated retroviruses and vaccines of the invention, additional elements in addition to endogenous NCTE which are essential for the virus' replication and/or pathogenicity can also be disabled or eliminated. For example, genetic engineering of a Nef-negative retrovirus can produce recombinant hybrid which can replicate *in vivo*, yet is less virulent. In the case of HIV-1, a functional Nef gene is important for development of high viremia and AIDS. Animals infected with Nef-deleted attenuated viruses are resistant to subsequent challenge with pathogenic wild-type viruses. A critical role for Nef in development of AIDS in humans has been suggested by the

observation that some individuals with a long-term nonprogressive HIV-1 infection (persons who show no clinical or immunological signs of immunodeficiency despite being HIV seropositive for over a decade) are infected with viruses carrying naturally occurring Nef deletions. The mechanism of Nef action remains incompletely understood, but multiple lines of evidence point out to a role in modulation of cellular signaling pathways via physical and functional interactions with host cell protein kinases. *In vitro* natural isolates of Nef down-regulate expression of the cell surface CD4 molecule, a component of the T cell antigen receptor and the viral receptor, by accelerating its endocytosis. Recombinant soluble Nef protein binds to cell surfaces of various murine lymphoid cell lines, including T and B lymphocytes and macrophages; cross-linking of the cell-bound Nef protein with anti-Nef antibodies induces apoptotic cytolysis of the cells. For a further description of Nef, see, *e.g.*, Saksela (1997) *supra*; Greenberg (1997) *supra*; Luo (1997) *J. Virol.* 71:9531-9537; Luo (1997) *J. Virol.* 71:9524-9530; Okada (1997) *supra*. Thus, the vaccine and retrovirus of the invention comprising a non-function Nef has incorporated an additional level of attenuation. To engineer a non-functional Nef, the skilled artisan can delete and or mutate any portion of the Nef coding sequence. Nef sequences are well known in the art, *e.g.*, see databases, such as the NCBI databases described above. For examples of HIV-1 Nef nucleic acid and polypeptide sequences, see, *e.g.*, Accession Nos. Y15123, U88826, Y15121, Y15120, Y15116, to name only a few.

## VI. DELIVERY OF NUCLEOTIDES INTO CELLS

The nucleic acids and oligonucleotides of the invention, including vectors expressing CTE<sub>IAP</sub> and IAP<sub>ORG</sub>, can be delivered into cells in culture, tissues and organisms for synthesis, mutation, screening and the like. For example, the invention provides for a method for screening for a post-transcriptional RNA nucleo-cytoplasmic transport element (NCTE) binding protein by contacting a CTE<sub>IAP</sub> of the invention with a test compound and measuring the ability of the test compound to bind the NCTE; and this screening technique can be used in intact cells. Inhibitory oligonucleotides of the invention, and vectors capable of expressing these sequences, are also transferred into intact cells in cell culture, tissues or intact organisms.

The nucleic acids and oligonucleotides of the invention can be transferred into a cell using a variety of techniques well known in the art. For example, oligonucleotides can be delivered into the cytoplasm spontaneously, without specific modification. Alternatively, they can be delivered by the use of liposomes which fuse with the cellular membrane or are endocytosed, *i.e.*, by employing ligands attached to the liposome, or attached directly to the oligonucleotide, that bind to surface membrane protein receptors of the cell resulting in endocytosis. For example, a DNA binding protein, *e.g.*, HBGF-1, is known to transport oligonucleotides into a cell. See, *e.g.*, Tseng (1997) *J. Biol. Chem.* 272:25641-25647; Satoh (1997) *Biochem. Biophys. Res. Commun.* 238:795-799, describing efficient gene transduction by Epstein-Barr-virus-based vectors coupled with cationic liposome and HVJ-liposome.

The procedures for delivering the nucleic acids and oligonucleotides of the invention to cells are useful *in vivo*. For example, by using liposomes, particularly where the liposome surface carries ligands specific for target cells, or are otherwise preferentially directed to a specific organ, one may provide for the introduction of the oligonucleotides into the target cells *in vivo*. See, *e.g.*, Huwyler (1997) *J. Pharmacol. Exp. Ther.* 282:1541-1546, describing receptor mediated delivery using immunoliposomes. Alternatively, cells may be permeabilized to enhance transport of oligonucleotides into the cell, without injuring the host cells. See, *e.g.*, Verspohl (1997) *Cell. Biochem. Funct.* 15:127-134; Kang (1997) *Pharm. Res.* 14:706-712; Bashford (1994) *Methods Mol. Biol.* 27, 295-305, describing use of bacterial toxins for membrane permeabilization; and for general principles of membrane permeabilization, see, *e.g.*, Hapala (1997) *Crit. Rev. Biotechnol.* 17:105-122.

## VII. PREPARATION, FORMULATION AND ADMINISTRATION OF ATTENUATED VIRAL VACCINES

Live, CTE<sub>IAP</sub>-attenuated HIV-1 virus can be grown and harvested from activated human peripheral mononuclear cells or from a variety of tissue culture cells, such as human 293 cell line, as described herein; see also, *e.g.*, Eberlein (1991) *Virus Res.* 19:153-161; Parente (1996) *Gene Ther.* 3:756-760; Margolis (1997) *AIDS Res. Hum. Retroviruses* 13:1411-1420. Virion-containing supernatants are collected, and, typically,

filtered. CTE<sub>IAP</sub> sequences in the harvested, attenuated virus for use in vaccine formulations can be confirmed by conventional sequencing. The attenuated virus can be further purified, for example, by ultrafiltration or ultra-centrifugation. The live, attenuated virus can be stored by refrigeration, or on a long-term basis, by freezing in liquid nitrogen.

5           A formulation for administering the virus as a vaccine is prepared using, for example, any physiologically acceptable buffer, such as saline or phosphate buffered saline (PBS). This can be stored in a frozen state. The formulation can also be freeze-dried, stored at room temperature, and reconstituted by adding appropriate volume of buffer. The vaccine pharmaceutical formulation can be in the form of a sterile injectable  
10           preparation, such as a sterile injectable aqueous or oleaginous suspension. This suspension can be formulated according to the known art using those suitable dispersing or wetting agents and suspending agents which have been mentioned above. The sterile injectable preparation can also be a sterile injectable solution or suspension in a nontoxic parenterally-acceptable diluent or solvent, such as a solution of 1,3-butanediol. Among the  
15           acceptable vehicles and solvents that can be employed are water and Ringer's solution, an isotonic sodium chloride. In addition, sterile fixed oils can conventionally be employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid can likewise be used in the preparation of injectables.

20           The live attenuated viral vaccine of the invention can be administered using any acceptable route, as, e.g., by application to a mucosal surface, by injection, by inhalation or by ingestion. For examples of inhalants, see Rohatagi (1995) *J. Clin. Pharmacol.* 35:1187-1193; Tjwa (1995) *Ann. Allergy Asthma Immunol.* 75:107-111; Fernandez-de Castro (1997) "Measles vaccination by the aerosol method in Mexico,"  
25           *Salud Publica Mex* 39:53-60. Injection of vaccine can be intravenous or intramuscular; see, e.g., Groswasser (1997) *Pediatrics* 100:400-403, as example of injection techniques for efficient intramuscular vaccine delivery. Administration by application to any mucosal surface, including, e.g., intraoral (sublingual, buccal, and the like), intranasal, intrarectal, intravaginal, or ocular. For examples of mucosal administration methods, see, e.g., Staats  
30           (1997) *AIDS Res Hum Retroviruses* 13:945-952; Okada (1997) *J. Immunol.* 159:3638-3647; Wu (1997) *AIDS Res Hum Retroviruses* 13:1187-1194.

The amount of virus (number of virions) per dose will vary depending on results of different titrations used in clinical trials. The range can range, for example, from only a few infectious units, to about  $10^4$  to  $10^{10}$  infectious units (*i.e.*, virions) per dose. Protocols and means to determine safety and efficacy used for other attenuated vaccines can be adapted and used with the novel reagents provided by the invention; see, *e.g.*, Gruber (1997) "Evaluation of bivalent live attenuated influenza A vaccines in children 2 months to 3 years of age: safety, immunogenicity and dose-response," *Vaccine* 15:1379-1384; Tingle (1997) "Randomised double-blind placebo-controlled study on adverse effects of rubella immunisation in seronegative women," *Lancet* 349:1277-1281; Varis (1996) "Efficacy of high-titer live attenuated varicella vaccine in healthy young children," *J. Infect. Dis.* 174:S330-S334; Gruber (1996) "Evaluation of live attenuated influenza vaccines in children 6-18 months of age: safety, immunogenicity, and efficacy," *J. Infect. Dis.* 173:1313-1319.

After the vaccine has formulated in an acceptable carrier, it can be placed in an appropriate container and labeled. For administration of the vaccine, such labeling would include, *e.g.*, instructions concerning the amount, frequency and method of administration. In one embodiment, the invention provides for a kit and instructional material teaching the indications, dosage and schedule of administration of the vaccine.

Selection of individuals who would benefit from receiving the live, attenuated vaccine of the invention include, but are not limited to, individuals who have a high risk of being exposed to HIV, such as intravenous drug users, individuals who may be exposed, as through a needle stick or transfusion, and individuals whose exposure to the virus has been confirmed, *e.g.*, by a positive blood test.

The vaccine can be administered in conjunction with other treatment regimens, *e.g.*, it can be coadministered or administered before or after any anti-viral pharmaceutical or a non-attenuated anti-HIV vaccine. The vaccine can be administered in any form of schedule regimen, *e.g.*, in a single dose, or, using several doses (*e.g.*, boosters) at dosages and time intervals to be determined by clinical trials.

The attenuated vaccine of the invention is considered efficacious, *i.e.*, immunoprotective, if it elicits any humoral or cell-mediated anti-HIV response without causing clinically significant pathology, such as significant T cell cytotoxicity. The anti-

HIV response can be assessed, for example, by measuring the levels of anti-viral antibodies or HIV-specific T cells, the amount of HIV in the blood or lymph nodes (see, e.g. Brown (1997) *Transfusion* 37:926-929), and/or levels of circulating helper (CD4<sup>+</sup>) T cells. See also, O'Brien (1997) "Changes in plasma HIV RNA levels and CD4<sup>+</sup> lymphocyte counts predict both response to antiretroviral therapy and therapeutic failure," *Ann. Intern. Med.* 126:939-945; Hughes (1997) "Monitoring plasma HIV-1 RNA levels in addition to CD4<sup>+</sup> lymphocyte count improves assessment of antiretroviral therapeutic response," *Ann. Intern. Med.* 126:929-938; Burgisser (1997) "Monitoring responses to antiretroviral treatment in human immunodeficiency virus type 1 (HIV-1)-infected patients by serial lymph node aspiration," *J. Infect. Dis.* 175:1202-1205.

#### VIII. SCREENING FOR NCTE BINDING PROTEINS USING CTE<sub>IAP</sub>

The invention provides for cell-based and *in vitro* assay systems to screen for novel NCTE-binding proteins using the CTE<sub>IAP</sub> of the invention. The full-length, two looped CTE<sub>IAP</sub> can be utilized, or, alternatively, a portion of a CTE<sub>IAP</sub>, such as a single loop A or a single loop B, can be used to assay for RNA binding proteins. One embodiment of the invention provides for a method of screening for an NCTE binding protein by contacting a CTE<sub>IAP</sub> of the invention with a test compound and measuring the ability of the test compound to bind the NCTE. Many assays are available that screen for nucleic acid binding proteins and all can be adapted and used with the novel reagents provided for by the invention. A few illustrative example are set forth below.

A variety of well-known techniques can be used to identify polypeptides which specifically bind to CTE<sub>IAP</sub>, for example, mobility shift DNA-binding assays, methylation and uracil interference assays, DNase and hydroxy radical footprinting analysis, fluorescence polarization, and UV crosslinking or chemical cross-linkers. For a general overview, see, e.g., Ausubel (chapter 12, DNA-Protein Interactions).

One technique for isolating co-associating proteins, including nucleic acid and DNA/RNA binding proteins, includes use of UV crosslinking or chemical cross-linkers, including cleavable cross-linkers dithiobis(succinimidylpropionate) and 3,3'-dithiobis (sulfosuccinimidyl- propionate); see, e.g., McLaughlin (1996) *Am. J. Hum. Genet.* 59:561-569; Tang (1996) *Biochemistry* 35:8216-8225; Lingner (1996) *Proc. Natl.*



*Aca. Sci. U.S.A.* 93:10712; Chodosh (1986) *Mol. Cell. Biol* 6:4723-4733. If a specific protein is believed to bind to CTE<sub>IAP</sub>, and an antibody is available or can be generated for that protein, co-immunoprecipitation analysis can be used. Alternatively, CTE<sub>IAP</sub>-affinity columns can be generated to screen for potential CTE<sub>IAP</sub>-binding proteins. In a variation of this assay, the CTE<sub>IAP</sub>-containing nucleic acid is biotinylated, reacted with a solution suspected of containing a CTE<sub>IAP</sub>-binding protein, and then reacted with a strepavidin affinity column to isolate the CTE<sub>IAP</sub>-containing nucleic acid/binding protein complex (see, e.g., Grabowski (1986) *Science* 233:1294-1299; Chodosh (1986) *supra*). The protein can then be conventionally eluted and isolated.

Mobility shift DNA-protein binding assay using nondenaturing polyacrylamide gel electrophoresis is an extremely rapid and sensitive method for detecting specific polypeptide binding to DNA (see, e.g., Chodosh (1986) *supra*, Carthew (1985) *Cell* 43:439-448; Trejo (1997) *J. Biol. Chem.* 272:27411-27421; Bayliss (1997) *Nucleic Acids Res.* 25:3984-3990). Interference assays and DNase and hydroxy radical footprinting can be used to identify specific residues in the nucleic acid protein-binding site, see, e.g., Bi (1997) *J. Biol. Chem.* 272:26562-26572; Karaoglu (1991) *Nucleic Acids Res.* 19:5293-5300. Fluorescence polarization is a powerful technique for characterizing macromolecular associations and can provide equilibrium determinations of protein-DNA and protein-protein interactions. This technique is particularly useful (and better suited than electrophoretic methods) to study low affinity protein-protein interactions, see, e.g., Lundblad (1996) *Mol. Endocrinol.* 10:607-612..

Proteins identified in by these techniques can be further separated on the basis of their size, net surface charge, hydrophobicity and affinity for ligands. In addition, antibodies raised against such proteins can be conjugated to column matrices and the proteins immunopurified. All of these general methods are well known in the art. See Scopes, R. K., *Protein Purification: Principles and Practice*, 2nd ed., Springer Verlag, (1987). Chromatographic techniques can be performed at any scale and using equipment from many different manufacturers (e.g., Pharmacia Biotech).

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be

suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

### EXAMPLES

5                   The following examples are offered to illustrate, but not to limit the claimed invention.

**Example 1: Identification, Isolation and Characterization of CTE<sub>IAP</sub>**

                  The following example details the identification, isolation and characterization of CTE<sub>IAP</sub>.

10                  To identify elements similar to SRV-1 CTE, as described by Tabernero (1996) *supra* and Zolotukhin (1994) *supra*, a search of the sequence databases for elements with nucleotide similarities to the 173 base pair SRV-1 NCTE was conducted. The RNA secondary structure of this NCTE element is a stem-loop structure with two internal loops, A and B. Queries were designated to search for homology to the loop motifs A and B,  
15                  which are separated by spacers to match the lengths of the interloop stem (15 to 35 nucleotides) and the hairpin loop: AANGACNGGT (2,1,1,) (SEQ ID NO:6); AACCTAAGACAGG (1,1,1) (SEQ ID NO:7); CNANGACNGG (2,1,1) (SEQ ID NO:8); and, AACCTAAGACAGG (1,1,1) (SEQ ID NO:9). The numbers in parentheses indicate the number of allowed nucleotide changes, deletions, and insertions, respectively. The  
20                  searches were performed with the PatScan program (<http://www.mcs.anl.gov/home/papka/ROSS/patscan.html>).

                  This search resulted in identification of a novel 176 base pair (bp) segment which has sufficient sequence identity to the submitted SRV NCTE element. This 176 bp sequence was a portion of a larger sequence which had been previously submitted as  
25                  GenBank accession number U53820. The novel element is located upstream of the coding region in the mouse osteocalcin related gene (ORG), as shown in Figure 2. ORG is a member of the murine osteocalcin gene cluster (see Desbois (1994) *J. Biol Chem.* 269:1183-1190; Rahman (1993) *Endocrinology* 133:3050-3053).

                  This novel 176 bp sequence was analyzed by computer program (using  
30                  MFold, University of Wisconsin Genetics Computer Group Package) for its ability to form secondary structures (MFold is an adaptation of the MFold package by Zuker and Jaeger,

see Zuker (1989) *Science* 244:48-52; Jaeger (1989) *Proc. Natl. Acad. Sci. USA* 86:7706-7710). The analysis predicted that the 176 bp sequence forms an RNA secondary structure that shares several important features with the NCTE structure predicted for SRV-1 CTE. As shown in Figure 1, two internal loops, designated loop A and loop B, are conserved. The distance between the two loops is also conserved. The AAGA "bulge" adjacent to loop A is conserved. To a lesser extent, the sequence of the hairpin loop is also conserved. Sequence comparison shows that the four stretches of imperfect direct repeats spanning internal loops A and B have significant sequence identity. In contrast, the stem structures show no sequence identity. Previous work has shown that the changing the sequence of the stem structure in SRV-1's NCTE does not affect function (Tabernero, 1996, *supra*). This finding, together with the lack of sequence identity between the stems of SRV and the 176 bp segment predicts that the stem structure serves a primarily structural, loop-separating role in the overall secondary structure.

The region upstream (5') of the novel segment was sequenced from the NCTE-containing plasmid, designated U53820, as described above. A computer sequence identity search using this new sequence data was performed. The analysis revealed that this NCTE is part of a novel IAP retroelement region within the mouse ORG. Thus, the novel CTE segment was named "CTE<sub>IAP</sub>" and the novel IAP termed "ORG<sub>IAP</sub>." As shown in Figure 2, the IAP is located in the transcribed ORG between its promoter and transcriptional start site and the ORG protein coding region (open reading frame).

The ORG<sub>IAP</sub> is 3,402 nucleotides in length and is flanked by two imperfect direct repeats, see Figure 2. The direct repeats have partial homology to the LTRs of the previously identified "Eker rat-associated IAP" (ERA-IAP) (Xiao (1995) *Oncogene* 11:81-87). Although they do not contain any intact open reading frames, ORG<sub>IAP</sub> LTRs contain a predicted TATA box, a polyadenylation signal, and a section with sequence identity to putative *gag/pol* and *env* regions of other IAPs. The novel CTE<sub>IAP</sub> of the invention is located between the putative *env* gene and the 3' LTR of ORG<sub>IAP</sub>. This is similar to the location of other NCTEs, as those seen in type D retroviruses.

**Example 2: CTE<sub>IAP</sub> Replacing HIV-1 NCTE Creates an Attenuated Virus**

The following example details the production of a Rev(-)/RRE(-) CTE<sub>IAP</sub>-containing HIV-1 hybrid recombinant, attenuated virus.

To demonstrate that the CTE<sub>IAP</sub> of the invention can produce an attenuated, immunoprotective HIV-1 vaccine, this novel NCTE element was inserted into a Rev/RRE-deficient recombinant clone of HIV-1, which is described in Zolotukhin (1994) *supra*, and is designated pR(-)Rev(-). Briefly, the HIV-1 molecular clone designated pNL4-3 (Adachi (1986) *J. Virol.* 59:284-291) was altered to lack functional Rev protein and Rev-responsive element (RRE), as described in Nasioulas (1994) *J. Virol.* 68:2986-2993, and Zolotukhin (1994) *supra*, respectively. 37 point mutations were introduced into the RRE that did not affect the overlapping env reading frame (Nasioulas (1994) *supra*). Two mutations that eliminated Rev were constructed. The first mutation eliminated the Rev initiation codon. The second mutation introduced a stop codon in the 23rd amino acid of the Rev protein (Zolotukhin (1994) *supra*).

A unique XhoI site was used to insert CTE<sub>IAP</sub> into the RRE and Rev deficient clone (pR(-)Rev(-)) at nucleotide 8887 of NL4-3, as described in Zolotukhin (1994) *supra*. This hybrid HIV-1 was termed "pR(-)Rev(-).IAP CTE." Stocks were prepared from: this hybrid; wild-type (wt) HIV-1 (the wt designated "NL4-3," Adachi (1986) *J. Virol.* 59:285-291); and, a R(-)Rev(-) hybrid into which was inserted an SRV-1 NCTE, termed "pR(-)Rev(-).S," (Zolotukhin (1994) *supra*). Human 293 cells were transfected by a calcium phosphate coprecipitation technique. Supernatants were collected and filtered through a 0.2 um filter. The sequence of CTE<sub>IAP</sub> in these replicating recombinant hybrids was confirmed by conventional sequencing techniques.

The wild-type NL4-3 and recombinant HIV-1 clones were used to infect Jurkat cells and *in vitro* activated human peripheral blood mononuclear cells (PBMCs) using a cell-free protocol, as described by Zolotukhin (1994) *supra*. PBMC cell cultures were obtained and activated as described in Zolotukhin (1994) *supra*. Briefly, PBMC cell cultures were obtained from healthy, HIV-seronegative blood donors, isolated by density centrifugation and stimulated for three days with phytohemagglutinin (PHA) at 5 to 10 micrograms per ml. Equal amounts of each of the viruses from the filtered 293 supernatants were used to infect  $5 \times 10^5$  activated PBMCs and  $3 \times 10^6$  Jurkat cells. Virus

propagation was monitored over time using a p24<sup>gag</sup> antigen capture assay (using either a commercial assay, Cellular Products (Buffalo, NY) or an in-house p24<sup>gag</sup> antigen capture assay using standard techniques, as described herein.

Replacement of HIV-1's wild type NCTE (RRE) by CTE<sub>IAP</sub> resulted in a slow-growing hybrid in both Jurkat and activated PBMCs, as demonstrated by Figure 3A and 3B, respectively. In PBMCs, the CTE<sub>IAP</sub>-containing hybrid, RRE(-)Rev(-).IAP CTE, as measured by p24 levels, replicated about 100 times less efficiently than the wild-type HIV-1 (NL4-3) and about 10 times less efficiently than the SRV-1 NCTE-containing hybrid, RRE(-) Rev(-).S. In Jurkat cells, the CTE<sub>IAP</sub>-containing hybrid replicated about 10 times less efficiently than the wild-type HIV-1 and the SRV-1 containing hybrid. This data demonstrates that CTE<sub>IAP</sub> attenuates HIV-1. Significantly, hybrid virus attenuated using the novel CTE<sub>IAP</sub> of the invention has a greater degree of attenuation than SRV-1 NCTE-attenuated HIV-1; CTE<sub>IAP</sub>-attenuated HIV-1 replicates about 10 times less efficiently than SRV-1 CTE-attenuated hybrids.

The role of the loop A structure was then investigated. A single nucleotide change was generated in CTE<sub>IAP</sub>'s stem structure immediately adjacent (5' end) to loop A. In CTE<sub>IAP</sub>, the GUC/GGC stem structure preceding loop A was changed to GCC/GGC, this new hybrid termed "pR(-)Rev(-).IAP CTE<sub>m</sub>." Creation of this G-C base pair (from the CTE<sub>IAP</sub> wild type U-G base pair) generates a stronger stem structure (three, rather than two, hydrogen bonds with the G-C bp). The SRV-1 CTE has the GCC/GGC stem structure. As seen in Figure 3, the "stronger" stem resulted in a hybrid which is less attenuated (*i.e.*, having less replicative potential), generating p24 antigen in amounts similar to the SRV-1 NCTE-containing hybrid.

CTE<sub>IAP</sub>'s replicative potential was further tested utilizing a syncytium formation assay. Human 293 cells were transfected with SRV-1 (with wild type CTE), a CTE negative SRV-1, and an SRV-1 clone which had had its CTE deleted and replaced with the CTE<sub>IAP</sub> of the invention. Transfectants were co-cultivated with the human Raji B cell line. The cultures were monitored for syncytium formation. SRV-1 wild type CTE and SRV-1/CTE negative clones reconstituted with the CTE<sub>IAP</sub> of the invention produced syncytium. CTE negative clones produced no syncytium.

In conclusion, hybrid HIV-1 using CTE<sub>IAP</sub> rather than the wildtype RRE or SRV-1 CTE as the NCTE element has less replicative potential (*i.e.*, is more attenuated), as shown by measuring levels of p24. Thus, these experiments demonstrate that the CTE<sub>IAP</sub> of the invention produces a slower-growing, attenuated HIV-1 hybrid.

**Example 3: CTE-Containing HIV-1 are Attenuated *In Vivo***

The following example details use of CTE-containing post-transcriptional regulatory agents in place of the wild-type Rev/RRE post-transcriptional regulatory system as attenuating agents in HIV-1 infection and AIDS pathogenesis. The efficacy CTE<sub>IAP</sub> as an HIV-1 attenuating agent *in vivo* can be demonstrated using functionally analogous NCTEs, such as the SRV-1 CTE.

Hybrid HIV-1 clones in which the NCTE from SRV-1 ("CTE") was inserted to replace the wild type HIV-1 NCTE (RRE) were used in the SCID-hu mouse model to demonstrate the replication and cytopathic effect of CTE(+) attenuated HIV-1. CTE(+) hybrids were used to infect a Thy/Liv implant (Kollmann (1995) *supra*) in SCID-hu mice. Significantly, CTE-attenuated viruses propagate slower than wild-type and Nef-negative (otherwise NCTE wild-type) HIV-1 clones. Significantly, as measured by HIV-1 proviral DNA levels (as measured by PCR in sequential biopsies), CTE-attenuated HIV-1 replicate about 10 times less efficiently than the SRV-1 NCTE-containing HIV-1.

Levels of circulating CD4<sup>+</sup> lymphocytes were monitored for 6 weeks after initial infection. No depletion of CD4<sup>+</sup> cells was observed. This demonstrates an attenuated phenotype for cytotoxicity of the CTE(+)/RRE(-)-containing HIV-1 clones. Direct comparison to a Nef-negative HIV-1 clone showed that the CTE(+)/RRE(-)-containing viruses are more attenuated, independent of the absence or presence of Nef. Therefore, the replacement of HIV-1's wild-type NCTE (RRE) with SRV-1 NCTE is responsible for the distinct, non-cytotoxic (non-CD4<sup>+</sup> lymphocyte depleting) phenotype of the slowly replicating hybrid HIV-1.

To distinguish between the effects of Rev and Nef on the HIV-1 viral replicative capacity and pathogenicity, the effects of Rev (-) and Nef (-) mutations on HIV-1 were compared. The recombinant HIV-1 clone into which the SRV-1 CTE had been inserted, termed "pR(-)Rev(-).S," as described in Example 2, was used. This hybrid is a

RRE(-)Rev(-)Nef(-) molecular clone of HIV-1 that contains the SRV-1 NCTE inserted within the *nef* gene. As discussed above, Rev and RRE were destroyed by multiple point mutations designed not to affect the overlapping *tat* and *env* open reading frames. To further distinguish between the effects of Rev and Nef on the viral phenotype, a Nef(+) version of the CTE-containing clone, R(-)Rev(-).S, was generated. For this, the N-terminal portion of *nef* was reinserted downstream of the SRV-1 CTE and the AUGs preceding the Nef initiation codon were destroyed. Specifically, the *nef* coding sequence was reconstructed in R(-)Rev(-).S. The proximal portion of *nef* spanning nucleotides 8782 to 8954 was PCR-amplified from HIV-1 NL4-3 using primers

gcatactcgagATAAGATGGGTGGCAAGTGG (sense) (SEQ ID NO:10) and GGCACAAGCAGCATTGTTAG (antisense) (SEQ ID NO:11). The regions of complementarity are shown in uppercase, while the lowercase sequence contains *Xho*I site that was used to insert the PCR fragment in the *Xho*I site of R(-)Rev(-).S. This resulted in pR(-)Rev(-)Nef(+)CTE that contains an intact *nef* gene downstream of the SRV-1 CTE.

To assure *nef* expression, all AUG codons within the remaining *nef* region upstream of CTE were converted to CUG by PCR mutagenesis. Two AUG codons within the 241 base pair SRV-1 CTE fragment that do not belong to the known minimal 173 base pair CTE were also mutated to CUG. The *Hpa*I-*Xba*I fragment was PCR-amplified from pR(-)Rev(-)Nef(+)CTE as two overlapping fragments, using primer pairs

ATAGTGCTGTAACTTGCTCAATGCCACAGCC (SEQ ID NO:12) and CTGCTCCCACCCCAgCTGCTGCTGGCTCAGCTCGTCTCAgTCTTTCCCTTACAGC AGGCCAgCCAATCACACTACTTTTGGACCACTTGCCACCCAgCTTATAGCAAAA TC (SEQ ID NO:13) for the 5'-portion; and GAGACGAGCTGAGCCAGCAGCAGcTGGGGTGGGAGCAG (SEQ ID NO:14) and

gcatacagctctagaCCAAGACAgCAgCCGGGCAGCACGG (SEQ ID NO:15) for the 3'-portion. The mutated nucleotides are shown in lowercase, and a flanking region containing the *Xba*I restriction site is italicized. These two PCR fragments were mixed and reamplified with primers ATAGTGCTGTAACTTGCTCAATGCCACAGCC (SEQ ID NO:16) and gcatacagctctagaCCAAGACAgCAgCCGGGCAGCACGG (SEQ ID NO:17), and the resultant fragment was used to replace the corresponding *Hpa*I-*Xba*I fragment of Rev(-)RRE(-)Nef(+)CTE(+). The structure of all recombinant regions was verified by

sequencing on both strands. All viral stocks and cultured cells were generated and prepared as described in Example 2.

Wild type HIV-1 (pNL4-3) and the CTE(+) clones pR(-)Rev(-)Nef(-)CTE (also called pR(-)Rev(-).S), and, pR(-)Rev(-)Nef(+)CTE (*i.e.*, pR(-)Rev(-).S with the Nef reinserted)) were transfected into human 293 cells by calcium phosphate coprecipitation. One day after the transfection, the cells were harvested and analyzed for viral protein expression. Western immunoblots (see Hadzopoulou (1989) *J. Virol.* 63:1265-1274) were incubated with HIV-1 patient serum and anti-Nef antibody. Nef protein was detectable only in cells transfected with the Rev-independent Nef(+) plasmid and wt HIV-1 (NL4-3). Both CTE(+) clones produced lower amounts of Env and Gag as compared to wild type virus. Quantitation of Gag protein expression using a p24<sup>gag</sup> antigen capture assay (see above) revealed about a 5-10-fold lower than wild-type levels of production by both of the CTE-containing clones. Thus, use of SRV-1 NCTE in place of wt HIV-1 NCTE results in a slow-growing virus, *i.e.*, a hybrid with a lower replicative capacity.

To further characterize the lower replicative potential of CTE-containing HIV-1, their infectivity in PHA-activated human PBMCs was determined by standard end-point dilutions (see McDougal (1985) *J. Immunol. Methods* 76:171-183). Briefly, viral aliquots were diluted in medium using 5-fold dilution steps starting with a 1:10 dilution. 100 microliters of Jurkat cell suspension was seeded in 96-well plates at  $7.5 \times 10^4$  cells per well. 100 microliters of virus dilution was added to 12 parallel wells. The culture medium was changed twice a week for the next two weeks and on day 14 a 120 microliter sample from each well was analyzed using the p24<sup>gag</sup> antigen ELISA assay described above. The ID<sub>50</sub> values were defined as the reciprocal of the virus dilution resulting in 50% positive wells using a Reed-Munch calculation (see Albert (1990) *AIDS* 4:107-112; McDougal (1985) *J. Immunol. Methods* 76:171-183). The ID<sub>50</sub> values for the CTE(+) clones were about 5-fold lower as compared to wild-type HIV-1. No significant difference in the infectivity was observed for the Nef(-) and Nef(+) variants of the CTE(+) viruses, indicating that RRE replacement, rather than Nef, is responsible for this slow-growing phenotype.

Next, propagation of the Nef(-) and the Nef(+) HIV-1 clones in human primary cells was determined. Human PBMCs were infected by equal infectious units of



the different viruses, using the viral stocks described above. Virus propagation was monitored over time by measuring extracellular Gag production (as described above). Wild-type HIV-1 (NL4-3) and NL4-3/Nef-negative clones had similar growth properties in activated PBMCs. The Nef(-) virus propagated approximately 5-10-fold less efficiently, whereas the replicative capacity of its Nef(+) counterpart was further decreased by about 5-fold. Protein production was also measured by Western blot analysis. Both p24<sup>gag</sup> and Western immunoblot measurements were comparable. Infection by the CTE(+) viruses in PBMCs resulted in lower levels of Gag and Env protein production, further demonstrating that replacement of HIV-1 RRE with a CTE affected both the infectivity and the replicative capacity of HIV-1.

The attenuation of the CTE-containing HIV-1 was further demonstrated by measuring its lower replicative capacity *in vivo* in an art-recognized animal model, the SCID-hu mouse (see Aldrovandi (1993) *Nature* 363:732-736; Bonyhaki (1993) *Nature* 363:728-732). The SCID-hu mice are produced by surgical implantation of human fetal liver and thymus under the kidney capsule of severe combined immunodeficient (SCID) mice. Normal T-cell differentiation has been shown to occur in the Thy/Liv implant. This is an art-recognized system for the study of HIV infection and cytotoxicity in human lymphopoietic tissue. Normally, infection by wt HIV-1 results in depletion of CD4<sup>+</sup> T cells. Significantly, infection with CTE-containing HIV-1, resulting in low levels of viral replication, did not cause CD4<sup>+</sup> cell depletion *in vivo*.

To further assess the attenuation of the CTE-containing HIV-1, virus load and cytotoxicity after infection in SCID-hu mice was measured. Virus stocks of wt HIV-1 (NL4-3), NL4-3/Nef negative, CTE(+) Nef(-), and CTE(+) Nef(+) HIV clones were tested. 1000 infectious units/mouse are typically used to establish good SCID-hu infection (*i.e.*, at least 50% to 90% infected) by attenuated strains of HIV-1, see Aldrovandi (1996) *J. Virol.* 70:1505-1511. Since the infectivity and replicative capacity of CTE(+) viruses are reduced (as demonstrated by the experiments described above), the amount of input virus was increased. The SCID-hu mice were infected by injection of virus into the Thy/Liv implant at 500 to 850 TCID<sub>50</sub> in a final volume of 100 µl. Sequential biopsies of the implants were performed 3 and 6 weeks postinfection, and the samples were analyzed for virus replication by quantitative DNA-PCR and for the number of CD4<sup>+</sup> thymocytes by flow

cytometry (PCR and cytometry as described in Aldrovandi (1996) *supra*). All 7 mice infected by wt HIV-1 scored positive for HIV proviral sequences at 3 weeks postinfection. Depletion of CD4<sup>+</sup> thymocytes (defined as measurement of less than 55% CD4<sup>+</sup> cells in the thymocyte population) was detected in 2 of 7 mice at this time point, and in another 2 of 2 mice analyzed after 6 weeks. None of the mock-infected mice showed any sign of HIV infection or CD4<sup>+</sup> thymocyte depletion. Replication of the NL4-3/Nef-negative was detectable in 2 of 10 mice after 3 weeks and in 8 of 9 mice at 6 weeks post-infection. Depletion of CD4<sup>+</sup> thymocytes was found in 3 of 9 mice infected with NL4-3/Nef-negative recombinants at 6 weeks postinfection (which is typical, as reported in Aldrovandi (1996) *supra*, and Jamieson (1994) *J. Virol.* 68:3478-3485).

In contrast, after infection with the CTE(+) Nef(-) and CTE(+) Nef(+) hybrids, provirus DNA was detectable only at the 6 week time point in 4 of 8 and 1 of 5 mice, respectively. The viral loads in the mice infected by the CTE(+) viruses were significantly lower than that of the RRE+/Nef-negative hybrids (*i.e.*, wt except for lack of Nef). While implants infected by the RRE+/Nef-negative hybrids showed 3,000 to 28,000 provirus copies per 10<sup>5</sup> cells, the CTE(+) Nef(-) virus contained about 500 to 2,400 copies per 10<sup>5</sup> cells, resulting in about a 20-fold decrease in average provirus copies in the CTE(+) infected mice. In CTE(+) Nef(+) virus only 1 of 5 mice was provirus positive after 6 weeks. Analysis of the implants of all the mice infected by CTE(+) viruses showed normal thymocyte profiles, *i.e.*, no cytotoxicity. The virus' ability to produce Nef did not affect pathogenicity. These findings suggest that replacement of wt RRE with an NCTE, in this case NCET from SRV-1, is primarily responsible for the slower growing, attenuated non-cytotoxic phenotype in the SCID-hu mice.

To address the question whether the lower virus load is responsible for the lower cytotoxicity of the attenuated, CTE(+) hybrids, the experiments were repeated using about 5-fold higher amounts of input viruses (approximately 2500 infectious units/mouse). Infection with these larger amounts of virus also does not lead to CD4<sup>+</sup> thymocyte depletion. New stocks for CTE(+) Nef(-) and CTE(+) Nef(+) HIV clones with higher titers were generated yielding 5x10<sup>4</sup> and 4.7x10<sup>4</sup> TCID<sub>50</sub>/ml, respectively. The Thy/Liv implants in SCID-hu mice were injected with 100 µl of these high titer stocks. Biopsies were analyzed at 3 and 6 weeks postinfection. At 3 weeks postinfection, no virus could be

detected in the 6 mice infected with the either of the CTE(+) viruses. At 6 weeks postinfection, in all 4 mice infected by the CTE(+)/Nef(-) hybrid and in all 7 mice infected by the CTE(+)/Nef(+) hybrid, HIV-1 replication was detectable. Although the time necessary to detect the CTE(+) hybrid was still 6 weeks, the viral loads were clearly elevated, due to the higher amount of input viruses. The average proviral copies per 10<sup>5</sup> cells was 15,000 in the case of CTE(+)/ Nef(-) hybrid, which is similar to that obtained from mice infected with 5-fold lower amount of RRE+/ Nef-negative clone. The virus load in the implants infected by the CTE(+)/Nef(+) variant was at least ten-fold lower than that of the CTE(+)/Nef(-) counterpart.

Interestingly, even with these differences in viral load, no significant changes were observed in the thymocyte profiles (within the time frame of the experiment, 6 weeks). Therefore, no depletion of CD4<sup>+</sup> thymocytes by CTE(+) hybrids was observed. Parallel infections using wt HIV-1 resulted in high levels of virus load accompanied by loss of CD4<sup>+</sup> cells. In conclusion, increasing the virus inocula did not increase the low cytotoxicity of the CTE(+) viruses *in vivo*, as observed in this SCID-hu mice model. Therefore, replacement of wt HIV-1 RRE with CTE generated hybrids that are less cytotoxic, more attenuated, than Nef-negative (otherwise wt) HIV-1.

In conclusion, using an NCTE functionally analogous to the CTE<sub>IAP</sub> of the invention, these data demonstrate the efficacy of NCTEs as attenuating agents in HIV-1 infection and AIDS pathogenesis *in vivo*. While CTE(+) HIV-1 hybrids can infect a Thy/Liv implant in SCID-hu mice, they propagate slower than wt HIV-1 and NL4-3/Nef-negative hybrids. No depletion of CD4<sup>+</sup> cells was observed in the CTE(+) hybrids, demonstrating an attenuated phenotype for cytotoxicity. Direct comparison to RRE+/Nef-negative HIV-1 showed that the CTE+ is responsible for attenuation independent of the absence or presence of Nef. Thus, these experiments demonstrate that NCTEs, such as the CTE<sub>IAP</sub> of the invention, can produce a slow-growing, attenuated HIV-1 hybrid *in vivo*.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference for all purposes.

**WHAT IS CLAIMED IS:**

1. An isolated nucleic acid consisting of a Constitutive Transport Element derived from an Intracisternal A Particle (CTE<sub>IAP</sub>) nucleotide sequence, the isolated CTE<sub>IAP</sub> nucleic acid defined as having the following properties:

5 (i) the CTE<sub>IAP</sub>, when inserted in a recombinant, hybrid HIV-1, is capable of functioning as a post-transcriptional RNA nucleocytoplasmic transport element (NCTE) in place of wild-type NCTE in the hybrid HIV-1, and when the CTE<sub>IAP</sub>-containing hybrid HIV-1 virus infects activated human peripheral blood mononuclear cells (huPBMCs), the level of expression of HIV-1 p24<sup>gag</sup> is between about 50 fold and about  
10 200 fold less than levels of p24<sup>gag</sup> expression when HIV-1 wild type virus, utilizing wild-type NCTE, infects activated huPBMCs; and,

(ii) the secondary structure of the CTE<sub>IAP</sub> comprises at least a two loop A domain and a loop B domain, wherein the nucleic acid primary sequence of the loops has at least 90% nucleic acid sequence identity, to a loop A and a loop B domain of a  
15 nucleic acid comprising the loop structure as set forth in Figure 1 and the sequence as set forth in SEQ ID NO:1; and, the distance between the loop A domain and the loop B domain is between about 11 to about 36 base pairs in length.

2. The isolated nucleic acid CTE<sub>IAP</sub> of claim 1, comprising a ribonucleic acid  
20 comprising a motif of contiguous base pairs consisting of:

X<sup>A</sup><sub>10-500</sub> - GUC AAUGAC GGGU AAGA - X<sup>B</sup><sub>16-24</sub> - ACCU AAGACA GG - X<sup>C</sup><sub>6-100</sub> - CAA  
U GUU - X<sup>D</sup><sub>6-100</sub> - CC GAGGAC AGGU - X<sup>E</sup><sub>16-24</sub> - CA ACCU AAGACA GGCA - X<sup>F</sup><sub>10-500</sub>

wherein X is any nucleotide base.

25 3. The isolated nucleic acid CTE<sub>IAP</sub> of claim 1, comprising a deoxyribonucleic acid comprising a motif of contiguous base pairs consisting of:

X<sup>A</sup><sub>10-500</sub> - GTC AATGAC GGGT AAGA - X<sup>B</sup><sub>16-24</sub> - ACCT AAGACA GG - X<sup>C</sup><sub>6-100</sub> - CAA T  
GTT - X<sup>D</sup><sub>6-100</sub> - CC GAGGAC AGGT - X<sup>E</sup><sub>16-24</sub> - CA ACCT AAGACA GGCA - X<sup>F</sup><sub>10-500</sub>

wherein X is any nucleotide base.

4. The isolated nucleic acid CTE<sub>IAP</sub> of claim 1 comprising the sequence as set forth in SEQ ID NO:1.

5. The isolated nucleic acid CTE<sub>IAP</sub> of claim 1 wherein the distance between the loop A domain and the loop B domain is between about 21 to about 26 base pairs in length.

6. An isolated transcription product of a CTE<sub>IAP</sub> nucleic acid, the CTE<sub>IAP</sub> nucleic acid defined as having the following properties:

(i) the CTE<sub>IAP</sub>, when inserted in a recombinant, hybrid HIV-1, is capable of functioning as a post-transcriptional RNA nucleo-cytoplasmic transport element (NCTE) in place of wild-type NCTE in the hybrid HIV-1, and when the CTE<sub>IAP</sub>-containing hybrid HIV-1 virus infects activated human peripheral blood mononuclear cells (huPBMCs), the level of expression of HIV-1 p24<sup>gag</sup> is between about 50 fold and about 200 fold less than levels of p24<sup>gag</sup> expression when HIV-1 wild type virus, utilizing wild-type NCTE, infects activated huPBMCs; and,

(ii) the secondary structure of the CTE<sub>IAP</sub> comprises at least a two loop A domain and a loop B domain, wherein the nucleic acid primary sequence of the loops has at least 90% nucleic acid sequence identity, to a loop A and a loop B domain of a nucleic acid comprising the loop structure as set forth in Figure 1 and the sequence as set forth in SEQ ID NO:1; and, the distance between the loop A domain and the loop B domain is between about 11 to about 36 base pairs in length.

7. An expression vector comprising a nucleic acid encoding a CTE<sub>IAP</sub> nucleic acid and a non-naturally occurring nucleic acid sequence, the CTE<sub>IAP</sub> nucleic acid defined as having the following properties:

(i) the CTE<sub>IAP</sub>, when inserted in a recombinant, hybrid HIV-1, is capable of functioning as a post-transcriptional RNA nucleo-cytoplasmic transport element (NCTE) in place of wild-type NCTE in the hybrid HIV-1, and when the CTE<sub>IAP</sub>-containing hybrid HIV-1 virus infects activated human peripheral blood mononuclear cells (huPBMCs), the level of expression of HIV-1 p24<sup>gag</sup> is between about 50 fold and about

200 fold less than levels of p24<sup>gag</sup> expression when HIV-1 wild type virus, utilizing wild-type NCTE, infects activated huPBMCs; and,

(ii) the secondary structure of the CTE<sub>IAP</sub> comprises at least a two loop A domain and a loop B domain, wherein the nucleic acid primary sequence of the loops has at least 90% nucleic acid sequence identity, to a loop A and a loop B domain of a nucleic acid comprising the loop structure as set forth in Figure 1 and the sequence as set forth in SEQ ID NO:1; and, the distance between the loop A domain and the loop B domain is between about 11 to about 36 base pairs in length.

8. A transfected cell comprising a polynucleotide encoding a CTE<sub>IAP</sub> nucleic acid and a non-naturally occurring nucleic acid sequence, the CTE<sub>IAP</sub> nucleic acid defined as having the following properties:

(i) the CTE<sub>IAP</sub>, when inserted in a recombinant, hybrid HIV-1, is capable of functioning as a post-transcriptional RNA nucleo-cytoplasmic transport element (NCTE) in place of wild-type NCTE in the hybrid HIV-1, and when the CTE<sub>IAP</sub>-containing hybrid HIV-1 virus infects activated human peripheral blood mononuclear cells (huPBMCs), the level of expression of HIV-1 p24<sup>gag</sup> is between about 50 fold and about 200 fold less than levels of p24<sup>gag</sup> expression when HIV-1 wild type virus, utilizing wild-type NCTE, infects activated huPBMCs; and,

(ii) the secondary structure of the CTE<sub>IAP</sub> comprises at least a two loop A domain and a loop B domain, wherein the nucleic acid primary sequence of the loops has at least 90% nucleic acid sequence identity, to a loop A and a loop B domain of a nucleic acid comprising the loop structure as set forth in Figure 1 and the sequence as set forth in SEQ ID NO:1; and, the distance between the loop A domain and the loop B domain is between about 11 to about 36 base pairs in length.

9. A recombinant retrovirus, wherein the retrovirus either lacks or has non-functional endogenous post-transcriptional RNA nucleo-cytoplasmic transport elements (NCTEs), further comprising a Constitutive Transport Element from an Intracisternal A Particle sequence (CTE<sub>IAP</sub>) operatively inserted into the retrovirus, the CTE<sub>IAP</sub> capable of acting as an exogenous functional NCTE to reconstitute the lacking or non-functional endogenous

NCTE and to reconstitute the infectivity of the retrovirus in a mammalian cell, the CTE<sub>IAP</sub> defined as having the following properties:

(i) the CTE<sub>IAP</sub>, when inserted in a recombinant, hybrid HIV-1, is capable of functioning as a post-transcriptional RNA nucleo-cytoplasmic transport element (NCTE) in place of wild-type NCTE in the hybrid HIV-1, and when the CTE<sub>IAP</sub>-containing hybrid HIV-1 virus infects activated human peripheral blood mononuclear cells (huPBMCs), the level of expression of HIV-1 p24<sup>gag</sup> is between about 50 fold and about 200 fold less than levels of p24<sup>gag</sup> expression when HIV-1 wild type virus, utilizing wild-type NCTE, infects activated huPBMCs; and,

(ii) the secondary structure of the CTE<sub>IAP</sub> comprises at least a two loop A domain and a loop B domain, wherein the nucleic acid primary sequence of the loops has at least 90% nucleic acid sequence identity, to a loop A and a loop B domain of a nucleic acid comprising the loop structure as set forth in Figure 1 and the sequence as set forth in SEQ ID NO:1; and, the distance between the loop A domain and the loop B domain is between about 11 to about 36 base pairs in length.

10. The recombinant virus of claim 9, wherein the virus is HIV-1.

11. The recombinant virus of claim 10, wherein the NCTE is RRE.

12. The recombinant virus of claim 10, wherein the HIV-1 further lacks a functional Nef.

13. A vaccine for the prophylaxis or amelioration of a viral infection in a mammal comprising an attenuated retrovirus,

wherein the attenuated retrovirus, when administered as a vaccine in sufficient amounts is capable of eliciting an immune response to the retrovirus in a mammal with a functional immune system,

wherein the attenuated retrovirus lacks an endogenous functional post-transcriptional RNA nucleo-cytoplasmic transport element (NCTE) and/or the ability to express an endogenous functional NCTE binding protein, and the attenuated retrovirus

further comprises a CTE<sub>IAP</sub> nucleic acid defined as having the following properties:

(i) the CTE<sub>IAP</sub>, when inserted in a recombinant, hybrid HIV-1, is capable of functioning as a post-transcriptional RNA nucleo-cytoplasmic transport element (NCTE) in place of wild-type NCTE in the hybrid HIV-1, and when the CTE<sub>IAP</sub>-containing hybrid HIV-1 virus infects activated human peripheral blood mononuclear cells (huPBMCs), the level of expression of HIV-1 p24<sup>gag</sup> is between about 50 fold and about 200 fold less than levels of p24<sup>gag</sup> expression when HIV-1 wild type virus, utilizing wild-type NCTE, infects activated huPBMCs; and,

(ii) the secondary structure of the CTE<sub>IAP</sub> comprises at least a two loop A domain and a loop B domain, wherein the nucleic acid primary sequence of the loops has at least 90% nucleic acid sequence identity, to a loop A and a loop B domain of a nucleic acid comprising the loop structure as set forth in Figure 1 and the sequence as set forth in SEQ ID NO:1; and, the distance between the loop A domain and the loop B domain is between about 11 to about 36 base pairs in length.

14. The vaccine of claim 13, wherein the attenuated retrovirus is HIV-1.

15. The vaccine of claim 14, wherein the attenuated HIV-1 further lacks a functional Nef.

16. The vaccine of claim 14, wherein the NCTE is RRE and the NCTE binding protein is Rev.

17. A kit for the prophylaxis or amelioration of a virus infection in a mammal, the kit comprising the vaccine of claim 13 and a pharmacologically acceptable carrier.

18. The kit of claim 17, further comprising an instructional material teaching the use of the vaccine, wherein the instructional material indicates that the vaccine is used for the prophylaxis or amelioration of HIV-1 infection in a mammal; that the vaccine is to be administered to a mammal in a therapeutically effective amount sufficient to express a viral protein; wherein the vaccine will not cause clinically significant CD4<sup>+</sup> cell depletion;



and, the expression of the viral protein elicits an immune response to the attenuated HIV-1 virus.

19. The use of a CTE<sub>IAP</sub> in the manufacture of a medicament for the prophylaxis or amelioration of a viral infection wherein the CTE<sub>IAP</sub> is defined as having the following properties:

(i) the CTE<sub>IAP</sub>, when inserted in a recombinant, hybrid HIV-1, is capable of functioning as a post-transcriptional RNA nucleo-cytoplasmic transport element (NCTE) in place of wild-type NCTE in the hybrid HIV-1, and when the CTE<sub>IAP</sub>-containing hybrid HIV-1 virus infects activated human peripheral blood mononuclear cells (huPBMCs), the level of expression of HIV-1 p24<sup>gag</sup> is between about 50 fold and about 200 fold less than levels of p24<sup>gag</sup> expression when HIV-1 wild type virus, utilizing wild-type NCTE, infects activated huPBMCs; and,

(ii) the secondary structure of the CTE<sub>IAP</sub> comprises at least a two loop A domain and a loop B domain, wherein the nucleic acid primary sequence of the loops has at least 90% nucleic acid sequence identity, to a loop A and a loop B domain of a nucleic acid comprising the loop structure as set forth in Figure 1 and the sequence as set forth in SEQ ID NO:1; and, the distance between the loop A domain and the loop B domain is between about 11 to about 36 base pairs in length.

20. The use of claim 19, wherein the viral infection is an HIV-1 infection.

21. A method for eliciting an immune response to a virus in a mammal, comprising administering to a mammal a therapeutically effective amount of an attenuated recombinant virus, wherein the virus comprises a CTE<sub>IAP</sub> defined as having the following properties.

(i) the CTE<sub>IAP</sub>, when inserted in a recombinant, hybrid HIV-1, is capable of functioning as a post-transcriptional RNA nucleo-cytoplasmic transport element (NCTE) in place of wild-type NCTE in the hybrid HIV-1, and when the CTE<sub>IAP</sub>-containing hybrid HIV-1 virus infects activated human peripheral blood mononuclear cells (huPBMCs), the level of expression of HIV-1 p24<sup>gag</sup> is between about 50 fold and about

200 fold less than levels of p24<sup>gag</sup> expression when HIV-1 wild type virus, utilizing wild-type NCTE, infects activated huPBMCs; and,

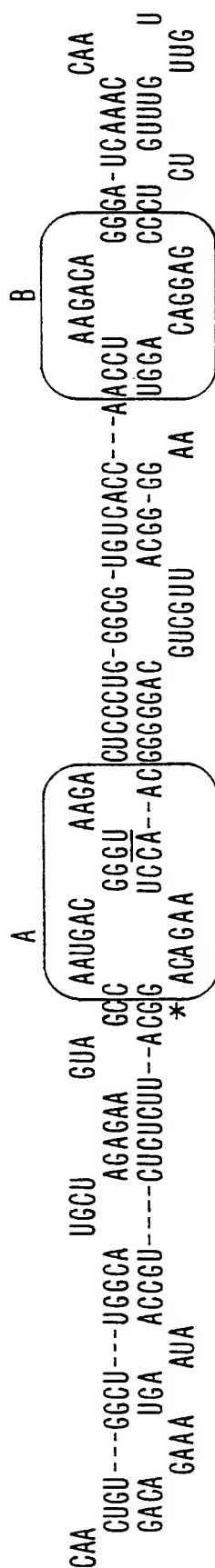
(ii) the secondary structure of the CTE<sub>IAP</sub> comprises at least a two loop A domain and a loop B domain, wherein the nucleic acid primary sequence of the loops has at least 90% nucleic acid sequence identity, to a loop A and a loop B domain of a nucleic acid comprising the loop structure as set forth in Figure 1 and the sequence as set forth in SEQ ID NO:1; and, the distance between the loop A domain and the loop B domain is between about 11 to about 36 base pairs in length.

22. A method for screening for a post-transcriptional RNA nucleocytoplasmic transport element (NCTE) binding protein comprising the following steps:

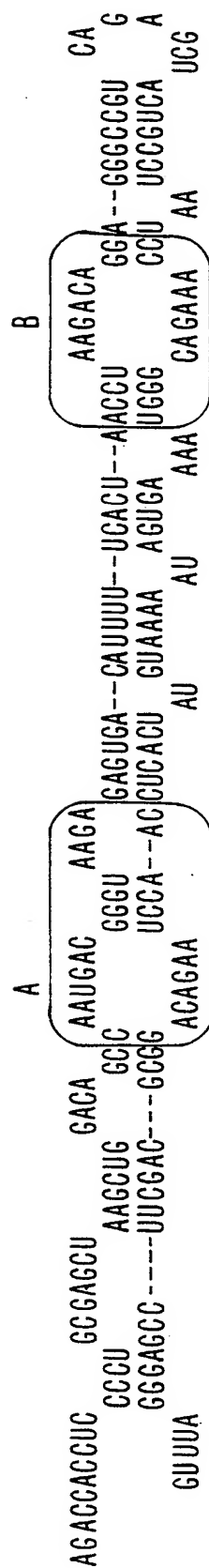
a) providing a composition comprising a loop A and/or a loop B of a CTE<sub>IAP</sub> ;

b) contacting the composition with a test compound; and,

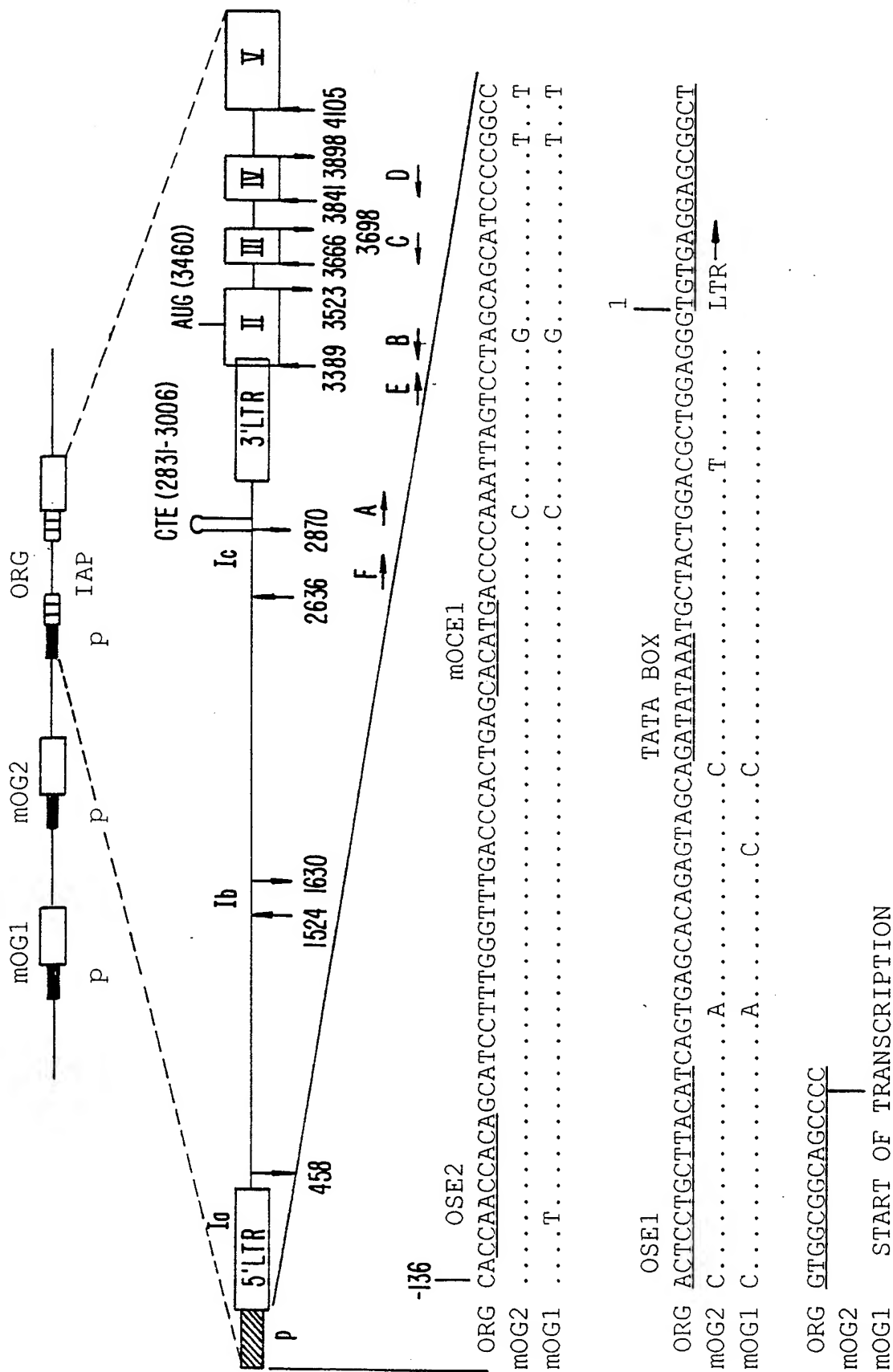
c) measuring the ability of the test compound to bind the NCTE.



**FIG. 1A.**



**FIG. 1B.**



**FIG. 2.**

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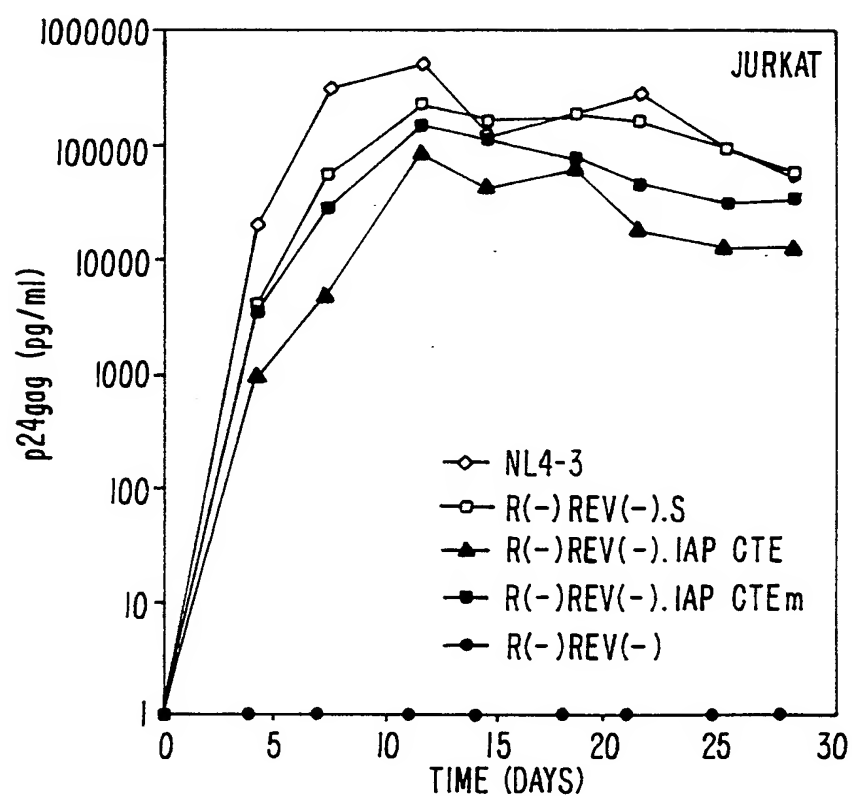


FIG. 3A.

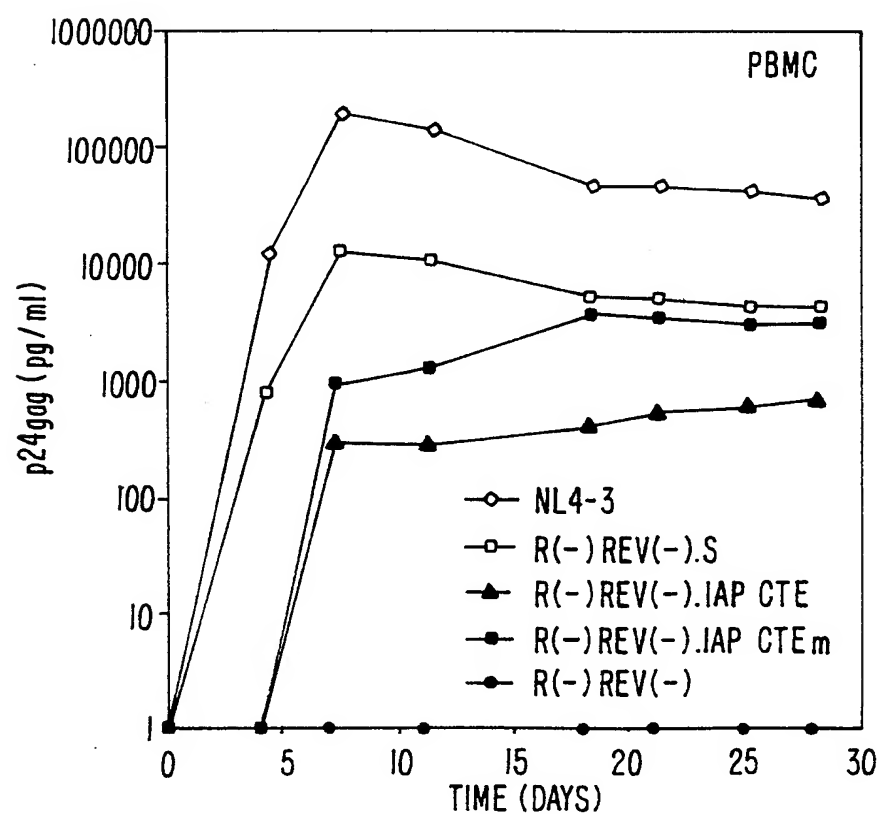


FIG. 3B.

**FIGURE 4** 4/5

PAGE 1 OF 2

SEQ ID NO:3

LOCUS MMU53820 4564 bp DNA

Mus musculus intracisternal A-particle-related retroelement ORG-IAP gene sequence.

GeneBank Accession No: U53820

SOURCE: house mouse; ORGANISM Mus musculus.

BASE COUNT 1118 a 1023 c 1236 g 1186 t 1 others

ORIGIN

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5/5

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3721 ggtatgtgtc ctctgggtc atttcttgg gtgactacct cctgaaggtc tcacaatctg  
3781 ctttgggatg gcagagggga agggacaaca catgaggag acagcaggga ggaaacagaa  
3841 ctaactacct gtttcttta cagatgccac gccaccggc cctgagtctg acaaaggtag  
3901 tggcagaaag cctggcaggg cctcggttg gccacacct gtcccctaag ccccaaatc  
3961 ccttgcctt ctgcctgggt gtcccactt ttctcactg aactcagaat tacctgacct  
4021 tgtgtgtctt ctccacagcc ttcatgtcca agcaggaggg caataaggta gtgaacagac  
4081 tccggcgcta ctttgggtta gtgccagagc ccttagcctt ccatattggt agggaggagt  
4141 tgttctgggg tagtctctat gaccgcaga gggctacacg tgcaggtcaa tccccagtc  
4201 caggaccttg gagcctcttg tacagtgtgg gaagaggatg tgtgtacccc tgtgtatatt  
4261 aatgccactg tgtgttggt gatgttactt tatgttctc agagcttcag tccccagccc  
4321 agatccccctg gagcccaccc gggagctatg tgagcttgac ccctgctgtg gacgagctat  
4381 caaaccagta tggtttaaag accgcctaca gacgcacta cggatcact atttaggacc  
4441 thtagctgcc cttaaagcaa actctggcag ctgggcttg gctgctctc cagaactga  
4501 cctccccctg tccctctctc tctgccctg caagtatgga tgtcacagca cgctccaaaa  
4561 taaa

//

(SEQ ID NO:3)

**FIG. 4.**

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/27792

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/48 C07K14/15 C12N7/01 A61K39/21 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TABERNERO C ET AL: "Identification of an RNA sequence within an intracisternal-A particle element able to replace Rev-mediated posttranscriptional regulation of Human Immunodeficiency Virus type 1" JOURNAL OF VIROLOGY, vol. 71, no. 1, January 1997, pages 95-101, XP002104206	1-12
A	AMERICAN SOCIETY FOR MICROBIOLOGY US see the whole document -----	13-22



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

### ° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

28 May 1999

Date of mailing of the international search report

11/06/1999

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
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Authorized officer

Cupido, M



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/ 27792

### Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claim 21  
is directed to a method of treatment of the human/animal  
body, the search has been carried out and based on the alleged  
effects of the composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such  
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all  
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment  
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report  
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is  
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

#### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.